

Tilapia Protein Hydrolysate Enhances Transepithelial Calcium Transport in Caco-2 cells

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ABSTRACT

Background: Adequate calcium intake is essential for calcium balance and normal health. Prolonged deficiency of calcium is associated with osteoporosis, dental changes, cataracts, and alterations in the brain. However, calcium is difficult to be directly absorbed from food due to the insoluble calcium salt precipitation that occurs in the intestinal environment. The aim is to investigate TPH produced by Nile tilapia and the stimulation of TPH-calcium-binding activity.

Methods: Tilapia protein hydrolysate (TPH) was prepared by alcalase digestion. Calcium-binding activity was measured using calcium colorimetric assay; absorption was at 612 nm. The interaction between TPH and calcium was examined by spectroscopic analysis, ultraviolet absorption, and fluorescence measurement. TPH-calcium-binding stability in the human digestion system was evaluated by in vitro pepsin-pancreatin hydrolysis simulating human gastric and intestinal digestion. The effects of food components on TPH-calcium-binding activity was also analyzed. The enhancement of transepithelial calcium transport by TPH was determined by in vitro Caco2 epithelial cell-like monolayer.

Results: TPH produced from Nile tilapia (*Oreochromis niloticus*) exhibited calcium-binding activity. Peptides in the hydrolysate that contributed to calcium binding since the spectroscopic changes induced by calcium were characteristic of peptide bonds and tryptophan residues. The calcium binding of TPH was compatible with food matrices. Most food components including saccharides, amino acids, and vitamins showed either positive or no effects on calcium binding. The calcium binding of TPH was also stable in the simulated gastrointestinal digestion system. Pepsin and pancreatin did not considerably change the calcium-binding activity of TPH. Of note, TPH reduced precipitation of calcium by oxalate and phytate, the two most anti-nutritional factors present in green leafy vegetables. Finally, we showed that TPH significantly promoted transepithelial calcium transport in the Caco-2 cell permeability model.

Conclusions: Tilapia protein hydrolysate produced by alcalase digestion possessed calcium-binding activity and prevents precipitation of calcium by a mineral-chelating agent as well as enhanced transepithelial calcium transport in the Caco-2 cell. The result implicated the potential of TPH as a functional food ingredient for promoting calcium absorption.

Keywords: *Tilapia protein hydrolysate; Calcium binding peptides; Calcium absorption*

INTRODUCTION

Calcium is an essential micronutrient required for many important functions in the human body. Calcium is needed to maintain strong bones, for the muscles to move, and for nerves to send messages. Almost every activity requires calcium for proper functions [1]. Calcium is absorbed into the small intestine of mammals by two mechanisms: active and passive transports. Transcellular active transport happens largely in the duodenum and upper jejunum, whereas passive calcium transport functions throughout the intestine [2]. When the calcium content of the chyme is low, a large portion of calcium is absorbed by the transcellular active transport process. However, when the mucosal calcium concentration exceeds 5 mM, transcellular active transport is downregulated and then accounts for only a small portion of calcium uptake [3]. The passive calcium transport process does not use energy. The rate of this process can be regulated by epithelial cells via controlling the tight junction permeability to calcium [3]. However, calcium is difficult to be directly absorbed from the food due to the insoluble calcium salt precipitation that occurs in the intestinal environment [4]. Consequently, there have been strong interests in developing methods to enhance calcium uptake.

Several studies have shown that bioactive peptides can enhance calcium uptake [5, 6]. Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions and may ultimately influence health. Bioactive peptides are inactive within the sequence of their parent proteins but can be released by enzymatic hydrolysis during either gastrointestinal digestion or food processing. They usually contain 2-20 amino acid residues [7]. The studies showed that protein hydrolysate from several food sources contain calcium-binding peptides. These peptides are bioactive able to promote calcium uptake via epithelium absorption, such as casein phosphopeptides (CPPs) [8, 5, 9], chicken eggshell matrix proteins [10], and tilapia scale protein hydrolysate [6].

A recent study showed that the peptides derived from the hydrolysate of tilapia muscle could solubilize amorphous calcium phosphate [11]. The amount of calcium these peptides can solubilize

is similar to that of CPPs do. This suggests their potential applications for improving calcium uptake. However, it remains unknown whether these peptides can enhance the transepithelial absorption of calcium. There is also a lack of systematic understanding of how common food components may affect the calcium-binding abilities of these tilapia-derived peptides.

Tilapia farming is proving to play an important role in food security in many countries. Tilapia is nutritious and forms a healthy portion of a balanced diet: high in protein (16-25%) and low in fat (0.5-3.0%) [12]. Tilapia is also low in carbohydrates and sodium, and its fatty acid composition is mostly unsaturated [13]. Thus, recently there have been an increase in research interest in exploring tilapia as functional foods and nutraceuticals [14]. In this study, we isolated the tilapia protein hydrolysate (TPH) that exhibited calcium-binding activity. We performed a systematic analysis of the effects of common food components on its calcium binding. Using Caco-2 cells as intestinal permeability model, we showed that TPH can significantly stimulate transepithelial calcium absorption.

MATERIALS AND METHODS

Materials

Fresh tilapia (*Oreochromis niloticus*), 700-900 g/fish, was purchased from a local market in Songkhla, Thailand. Alcalase 2.4 L (EC number 3.4.21.62) was purchased from Novozyme (Bagsvaerd, Denmark). QuantiChrom Calcium Assay Kit DICA-500 was purchased from Bioassay System, USA. Food components used were of food grade. Heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2 cell line) were obtained from the American Type Cell Collection (ATCC, HTB-37). Minimum Essential Medium (MEM) Gibco were purchased from ATCC. Transwell permeable supports (12 mm² inserts, 0.4 µm pore size, polycarbonate membrane) were purchased from Corning Costar, UK. A MilliCell ERS-2 Volt ohmmeter used to measure Transepithelial Electrical Resistance (TEER) was made by Millipore, USA. Pancreatin and pepsin were purchased from Sigma-Aldrich (St Louis, MO, USA). All other reagents and chemicals used were of analytical grade.

Enzymatic hydrolysis of tilapia

300 g of fresh tilapia was cut into small pieces and minced by a meat grinder and homogenizer. The minced tilapia was mixed with deionized water in a ratio of 1:2 (w/v). The mixture was adjusted to pH 8.5 using 6 N NaOH and agitated for 15 min at room temperature. The mixture was then placed in a water bath to maintain at 55 °C before adding 1% alcalase (w/w). The mixture was hydrolyzed and agitated by an overhead stirrer from 0 to 360 min. The hydrolysis was stopped by heating the system at 95 °C for 15 min. Finally, the hydrolysate was centrifuged at 3000 x g for 20 min. The supernatant, referred to as TPH, was measured for protein content by the Lowry method, lyophilized, and then kept at -20 °C for further experiments. The 60 ± 2.5 g of lyophilized TPH powder contains 830 ± 10.6 mg protein/g powder.

Analysis of TPH-calcium binding activity

The calcium-binding activity of TPH was determined by the calcium colorimetric assay kit DICA-500. The lyophilized TPH was dissolved in deionized water and diluted with a buffer of 100 mM Tris-HCl pH 7.8. The dissolved TPH was mixed with CaCl₂ in the same buffer. The mixture was gently agitated at 37 °C for 1 hour and then centrifuged at 6,000 x g for 15 min. The calcium

content in the supernatant was determined using the calcium colorimetric assay kit. The absorbance at 612 nm was measured after adding the developing reagent into the reaction. The calcium-binding activity was calculated as follows: Calcium-binding activity (mg/ml) = $C_{total} - C_{free}$, where C_{total} was calcium concentration measured without TPH; C_{free} , calcium concentration measured after adding TPH subtracted by the calcium amount in TPH. To determine the effects of food components on TPH-calcium-binding, various concentrations of food components were added into the TPH-calcium mixtures and then the amounts of free calcium were measured.

Spectroscopic analysis of TPH-calcium interaction

The ultraviolet absorption spectra of TPH were recorded between the wavelengths 190 nm and 400 nm using a UV-visible spectrophotometer (Shimadzu, model UV-1601 PC). TPH was mixed with various concentrations of $CaCl_2$ in the buffer of 100 mM Tris-HCl pH 7.8. The mixtures were incubated for 1 hour before the spectra were recorded. Fluorescence of TPH in the absence and presence of $CaCl_2$ was measured using a spectrofluorometer (Jasco FP-8200). The excitation wavelength was 280 nm and emission was recorded between 300-450 nm.

Simulated gastrointestinal digestion system

An in vitro pepsin-pancreatin hydrolysis simulating human gastric and intestinal digestion was carried out according to previous studies [15, 16]. Lyophilized TPH containing 10 g proteins was mixed with 400 mL of distilled-deionized water. After the pH was adjusted to 2.0 with 1 M KCl-HCl, pepsin was added at the ratio of 145 U/g proteins. The mixture was incubated at 37 °C for 3 hours. Then, the sample was submerged in a boiling water bath for 10 min to deactivate pepsin. After pepsin hydrolysis, TPH was adjusted to pH 7.0 by 2 N NaOH. Pancreatin was then added into the hydrolysate at the ratio of 30 U/g proteins. The mixture was further incubated at 37 °C for 4 hours. Finally, the sample was submerged in a boiling water bath for 10 min to inactivate pancreatin. The digested TPH at various times were cooled down to room temperature and centrifuged at 6,000 x g for 15 min. The calcium content in the supernatant was measured using the calcium colorimetric assay kit. The peptide patterns of the hydrolysate were determined by Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Tricine-SDS-PAGE).

Cell culture

The Caco-2 human colon cancer cell line was obtained from the American Type Cell Collection. Cells were cultured in 75 cm² plastic flasks in the Minimal Essential Media (MEM) Gibco supplemented with 15% fetal bovine serum (FBS), 1% nonessential amino acids, 1.5% penicillin, and 1.5% streptomycin. Cells were maintained in a humidified incubator with 5% CO₂ at 37 °C and sub-cultured every 2 days or when the cells reached the confluence of 80-90%. Cells were detached from the flasks using 0.25% trypsin-EDTA (Gibco BRL).

Cell viability assay

Cell viability was measured by MTT assay [17, 18]. Briefly, Caco-2 cells were seeded at a density of 1×10^5 cells per well in 96-well cell culture plates. The cells were incubated at 37 °C in 5% CO₂ for 24 hours to allow them to become fully differentiated. Then, various concentrations of TPH were added into the cells, and the cells were incubated at 37 °C in 5% CO₂ for another 24 hours. Subsequently, MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium was added into

the cell cultures, and the cells were incubated at 37 °C for 3 hours in the darkroom until formazan crystals formed. After the medium was removed, DMSO was added into each well to dissolve the formazan crystals. After the cells were gently agitated for 10 min at 37 °C, absorbance was read at 570 nm using a microplate reader (BioTek, PowerXS, USA). Cell viability (%) was calculated as follows: Cell viability (%) = [Mean OD sample/OD control] x 100, where the control sample was performed without TPH.

Caco-2 cell monolayer

To generate a Caco-2 cell monolayer, the cells were seeded at a density of 2.6×10^4 cells per cm^2 into transwells. The culture media were added into both apical and basolateral chambers of the transwells. The cells were incubated at 37 °C in 5% CO_2 . The media were changed every 3 days for about 21 days or until the cells were fully differentiated as a monolayer as measured by transepithelial electric resistance (see below).

Transepithelial Electric Resistance Measurement

Monolayer integrity was measured by transepithelial electrical resistance (TEER) using the MilliCell ERS-2 Voltohmmeter. TEER values of 3 different positions per well were recorded every 3 days for about 21 days. When TEER values reached 400-500 $\Omega \cdot \text{cm}^2$, the cells were assumed to be fully differentiated as a monolayer and appropriate for nutrient or calcium transport studies. TEER measurement involves measuring the blank resistance (R_{blank}) of the semipermeable membrane only (without cells) and measuring the resistance across the cell layer on the semipermeable membrane (R_{total}) in units of Ω [19]. The cell-specific resistance (R_{tissue}) is obtained as $R_{\text{tissue}} (\Omega) = (R_{\text{total}} - R_{\text{blank}})$. The resistance is inversely proportional to the effective area (cm^2) of the semipermeable membrane (M_{area}). TEER values are typically reported in units of $\Omega \cdot \text{cm}^2$ and calculated as: $\text{TEER} = R_{\text{tissue}} (\Omega) \times M_{\text{area}} (\text{cm}^2)$. The area of the insert membrane (M_{area}) in this study is 1.12 cm^2/well .

Calcium transport across Caco-2 cell monolayer

Calcium transport experiments were performed using Caco-2 cell monolayers in transwells with a transport buffer containing (mM) 140 NaCl, 5.8 KCl, 0.34 Na_2PO_4 , 0.44 KH_2PO_4 , 0.8 MgSO_4 , 20 N-Z-hydroxyethyl piperazine-N'-2-ethane sulfonic acid, 4 glutamine, and 25 D-glucose (pH 7.4). The transwell inserts were placed in 12-well culture plates. Various concentrations of calcium and/or TPH in the transport buffer were applied into the apical chambers of the transwells. The transport buffer only was added to the basolateral chambers. The transwell plates were gently agitated in a shaking incubator at 37 °C for 150 min. An aliquot of the samples in the basolateral chambers was collected every 30 min from 0 to 150 min for the determination of calcium content. The calcium concentration was determined using the calcium assay kit as described previously. The efficiency of calcium transport was expressed as the permeability coefficient (Papp) [20] which was calculated according to the equation: Permeability coefficient (Papp) (cm/s) = $(dQ/dt)/(C_0 \times A)$, where dQ/dt is the amount of calcium in basolateral chamber as a function of time ($\mu\text{mol/s}$); C_0 , the initial concentration of calcium in apical chamber (mol/L); A, the area of filter insert (1.12 cm^2).

Statistical analysis

All experiments were done in triplicate. Data were expressed as mean \pm SD. The differences among the groups were analyzed by one-way ANOVA followed by Duncan's multiple-comparison

test using SPSS software (version 22.0, IBM). The p-value of less than 0.05 was considered to be significant. In Figures, mean values with different letters were significantly different and values with the same letters not significantly different.

RESULTS AND DISCUSSION

Production of calcium-binding tilapia protein hydrolysate (TPH)

Tilapia protein hydrolysate (TPH) was generated by incubating minced tilapia with Alcalase. The hydrolysate was measured for calcium binding at different time intervals during the hydrolysis (Figure 1). The calcium-binding activity increased by more than two-fold for the first 30 min of hydrolysis. The highest calcium binding activity was found at 240 min. There was no significant activity change between 270 and 360 min. This result is consistent with our previous study that the calcium-binding activity of TPH was dependent on the time of hydrolysis [11]. The hydrolysate at 240 min of hydrolysis was selected for further experiments.

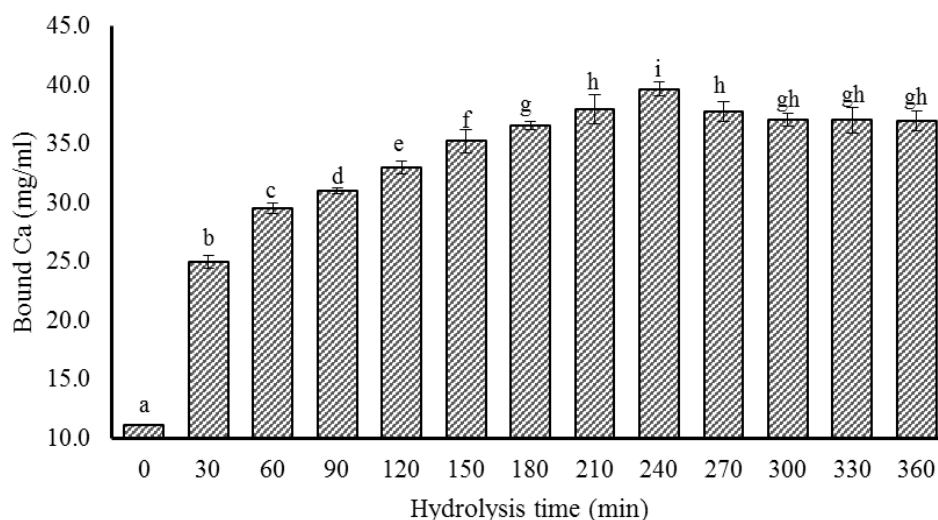


Figure 1. TPH calcium-binding activities at different hydrolysis times. Mean values with different letters were significantly different ($p < 0.05$).

Spectroscopic characterization of TPH-calcium interaction

To confirm the interaction between TPH and calcium, spectroscopic analysis of TPH in the presence of various concentrations of CaCl_2 was evaluated (Figure 2). In ultraviolet spectroscopy, the strongest absorption peaks were observed in the range of 210-236 nm (Figure 2A). This absorption is characteristic of peptide bonds and their carbonyl groups. At no or low calcium concentrations (0-2.5 mM), the strongest absorption peaks were found at 229 nm, whereas the peaks shifted to 226 nm when calcium concentration increased (Figure 2B). Increased calcium concentration also increased the absorbance of the maximum absorption peaks from 3.50 to 3.95. The calcium displayed hyperchromic and hypsochromic effects on TPH absorption which indicated an interaction. The absorption of the peptide bond is due to the transition of carbonyl electrons from a ground state to excited states ($n \rightarrow \pi^*$). Different transitions occur when the spatial structure of peptide chromospheres ($\text{C}=\text{O}$ and $-\text{COOH}$) or auxochromes ($-\text{OH}$ and $-\text{NH}_2$) is disturbed by binding [21]. Changes in absorption spectra often indicate that the oxygen atom of the carbonyl group and nitrogen of the amino group in the peptide bond are involved in interaction

[22]. This supported that the blue shift of TPH absorption caused by calcium indicated the interaction between TPH and calcium.

Tryptophan fluorescence of TPH was also affected by calcium (Figure 2C). At the emission maximum of 351 nm, the calcium showed biphasic effects on fluorescence intensity. At low calcium concentrations (0-5 mM), fluorescence emission was increased in quantum yield when the calcium concentration increased. At high calcium concentrations (5-10 mM), however, increasing calcium concentration was accompanied by a decrease in fluorescence intensity. Of note, the calcium concentration at the biphasic turning point coincided with that at the spectral shift point of TPH absorption (Figure 2B). Calcium binding has been shown to cause fluorescence quenching of peptides [23]. Bound calcium might cause the peptide to fold as a more compact structure contributing to the change in fluorescence intensity. [24]

The addition of calcium to TPH caused the peptide-characteristic changes in its absorption spectra and intrinsic tryptophan fluorescence. This suggested that it was the peptides in the hydrolysate that contributed to the calcium-binding activity. Calcium binding by intact proteins is well studied. The most representative examples are EF-hand proteins in which calcium-binding is mediated primarily by the carboxyl side chains and main chain carbonyl oxygens [25]. However, the exact modes of peptide binding to calcium have not yet been elucidated. Previous studies showed that the negatively charged peptides showed greater binding to calcium than the positively charged peptides [6]. The length and amino acid composition of peptides could also influence calcium-binding activities [26].

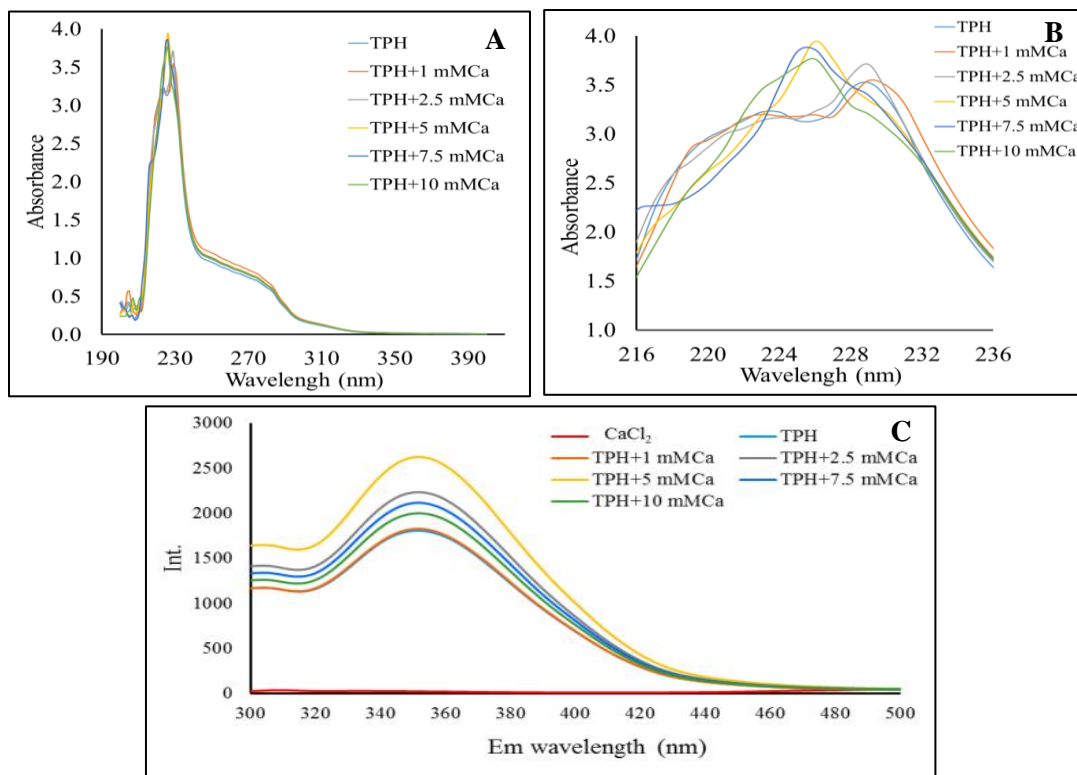


Figure 2. Spectroscopic analysis of TPH-calcium-binding. (A) UV spectra were recorded in the wavelength range of 190-400 nm in the presence of TPH and various calcium concentrations. (B) Enlarged view of the UV wavelength range 216-236 nm. (C) Fluorescence spectra of TPH were recorded with the excitation wavelength 280 nm and emission wavelength range 300-450 nm in the presence of various calcium concentrations.

Effect of food components on TPH-calcium binding

Foods are complex mixtures containing a large number of components. Some food components could potentially affect the calcium-binding activity of TPH. We assayed the amount of calcium sequestered in the presence of both TPH and food components including saccharides, amino acids, fatty acids, vitamins, minerals, and chelating agents (Figures 3-6). We found that neither monosaccharides (glucose, fructose, and galactose) nor disaccharides (lactose and sucrose) affected the level of bound calcium (Figure 3).

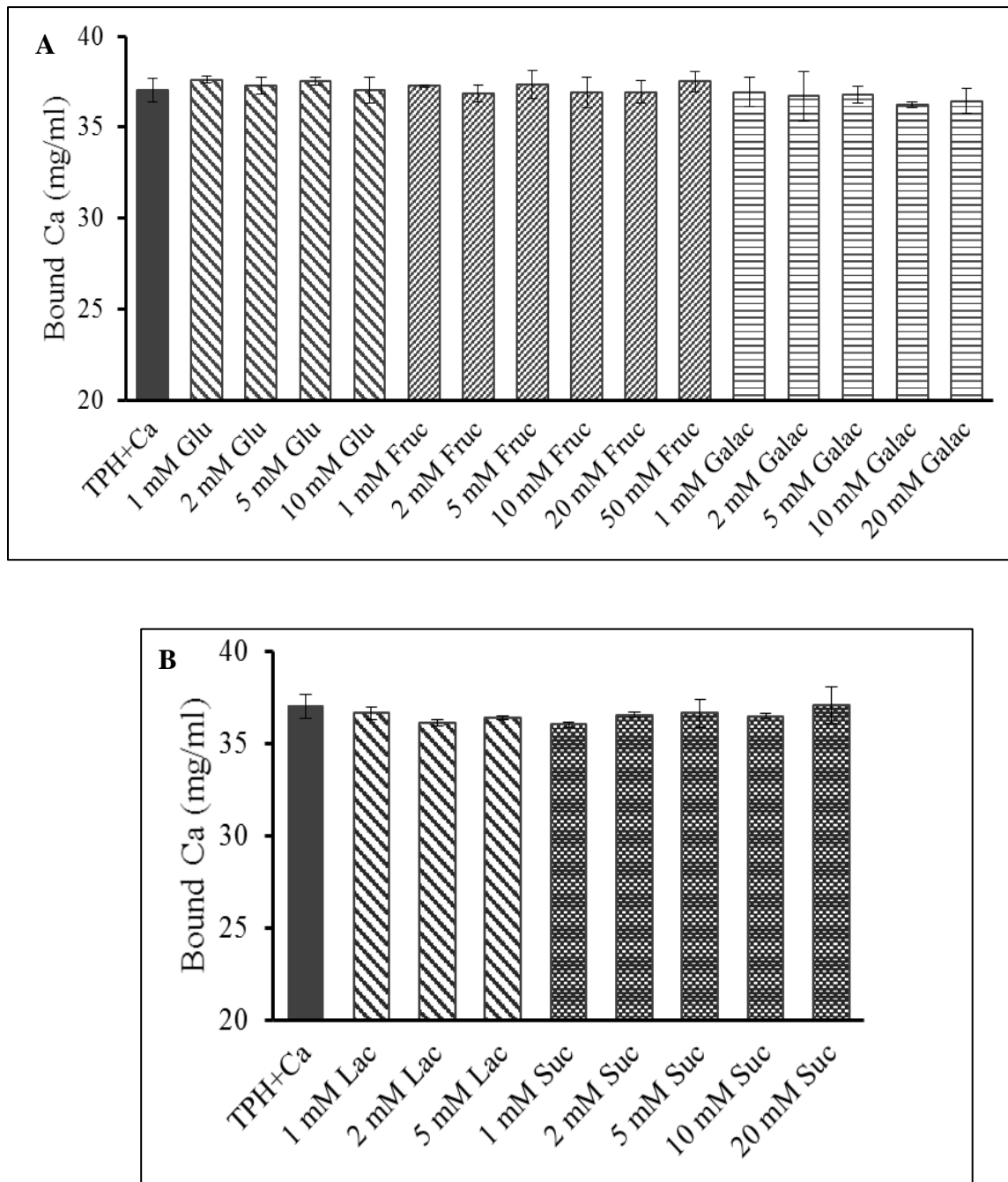


Figure 3. Effects of saccharides on TPH-calcium-binding. (A) Monosaccharides: glucose, fructose, and galactose. (B) Disaccharides: lactose and sucrose. Mean values with the same letters were not significantly different ($p > 0.05$).

For amino acids, calcium-binding was not affected by leucine, valine, or threonine (Figures 4A-C) but was significantly increased in the presence of aromatic residues (phenylalanine and tryptophan) or charged residues (lysine, histidine, aspartic acid, and glutamic acid) as shown in Figures 4D-I. This indicated the effect of being amino-acid specific. However, the latter amino acids did not affect the calcium level in the absence of TPH, suggesting that their effect was TPH dependent.

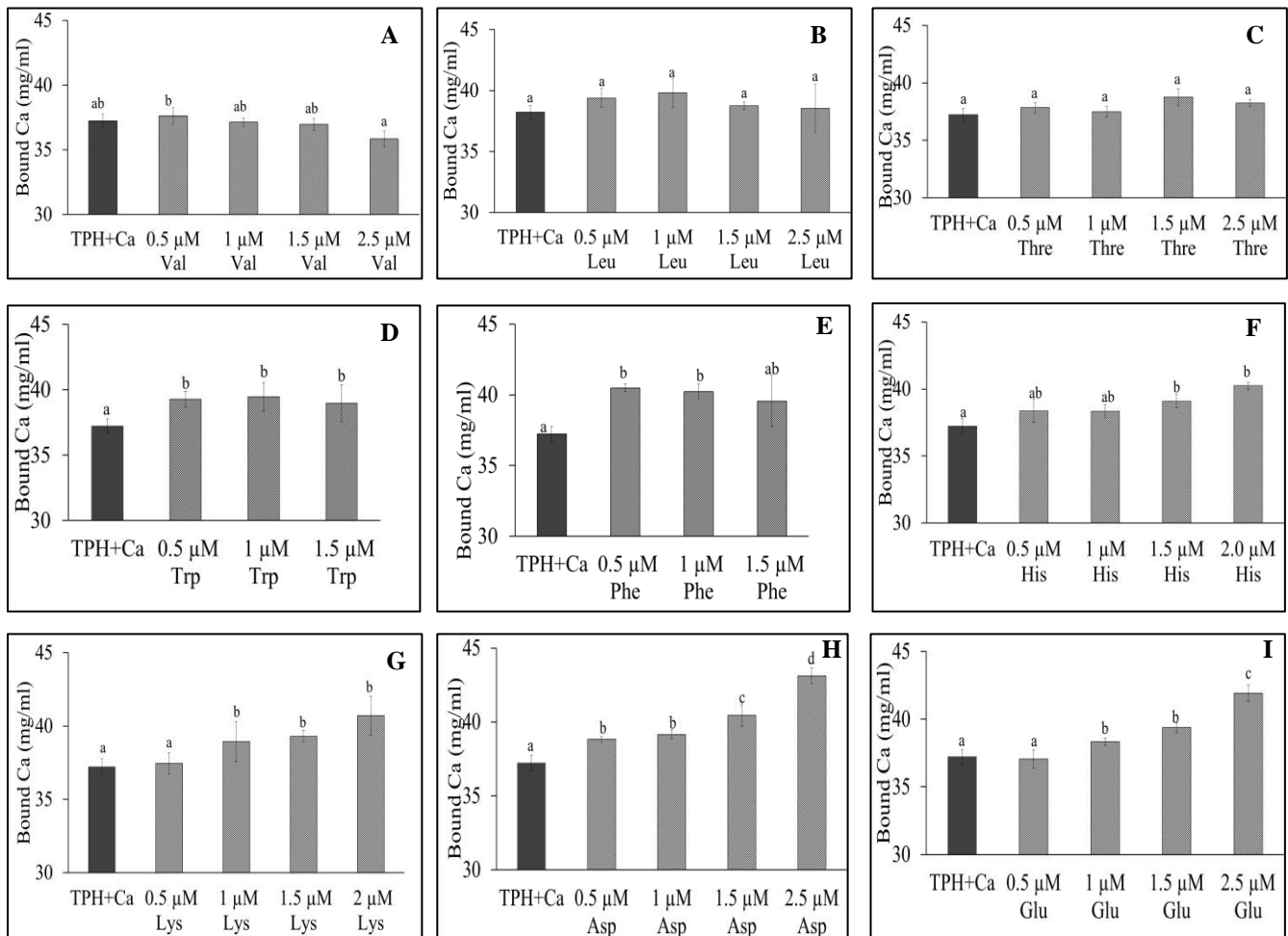


Figure 4. Effects of amino acids on TPH-calcium-binding. (A) Valine. (B) Leucine. (C) Threonine. (D) Tryptophan. (E) Phenylalanine. (F) Histidine. (G) Lysine. (H) Aspartic acid. (I) Glutamic acid. Mean values with different letters were significantly different ($p < 0.05$).

Fatty acids including linoleic acid and linolenic acid negatively affected calcium-binding (Figure 5A). While vitamin C slightly increased the amount of bound calcium, vitamin B did not show any significant effect (Figures 5B-C). Vitamin C might directly bind to calcium as its two hydroxyl groups were able to chelate calcium in an aqueous solution [27]. Minerals including magnesium, potassium, and sodium did not make an effect on calcium binding, but the effects of iron were concentration-dependent (Figures 5D-G). The calcium-binding activity significantly increased when adding 1 or 2 mM of FeCl_2 but decreased at 10 mM of FeCl_2 (Figure 5G).

The most anti-nutritional factors present in green leafy vegetables are oxalate and phytate which bind calcium making it insoluble and decreasing calcium bioavailability [28]. We found that TPH prevented calcium from being precipitated by these chelating agents (Figure 6). Without TPH, free calcium was reduced by more than 50% by 1 mM of oxalic acid, whereas no

considerable difference was caused in the presence of TPH (Figure 6A). Similar results were found for phytic acid in which TPH protected calcium from precipitation (Figure 6B). This result suggested that TPH might inhibit calcium chelating by oxalic acid and phytic acid.

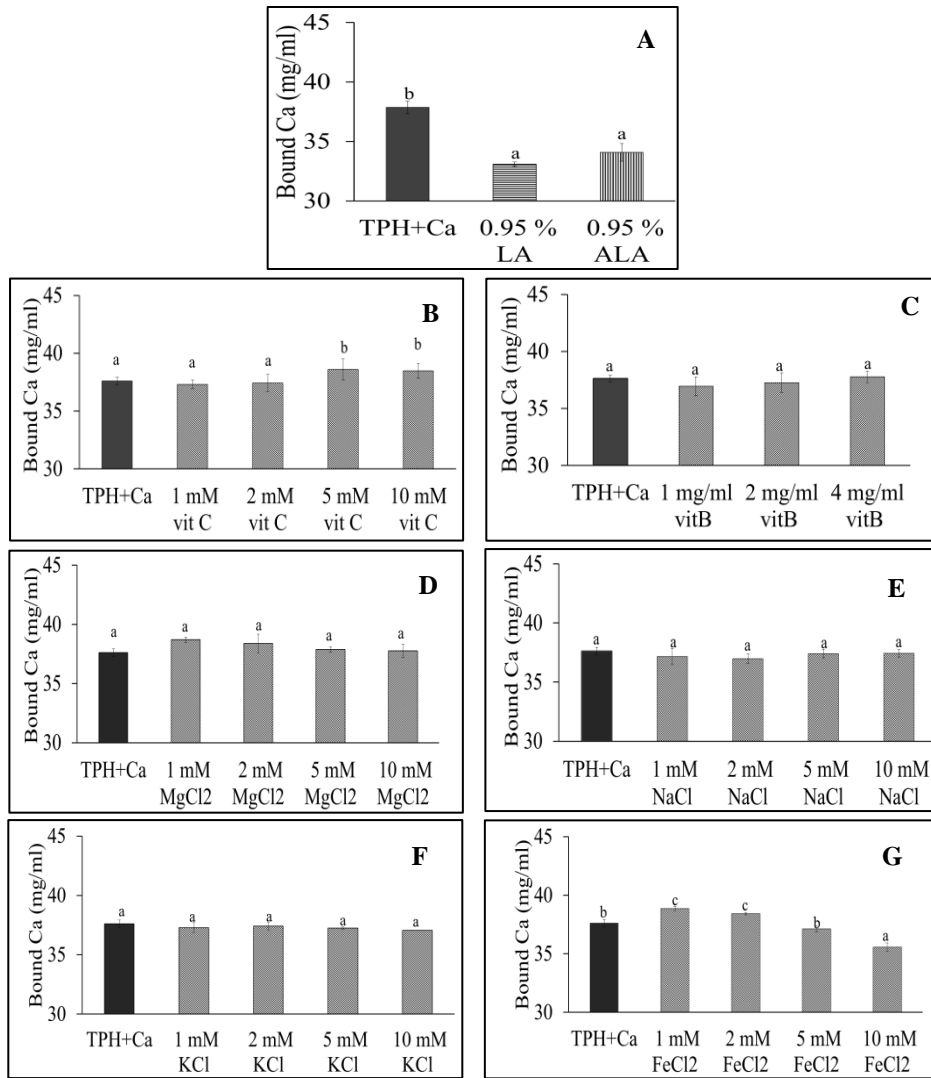


Figure 5. Effects of essential fatty acids, vitamins and macro minerals on TPH-calcium-binding. (A) Linoleic acid and linolenic acid. (B) Ascorbic acid (vitamin C). (C) Vitamin B. (D) MgCl₂. (E) NaCl. (F) KCl. (G) FeCl₂. Mean values with different letters were significantly different (p < 0.05).

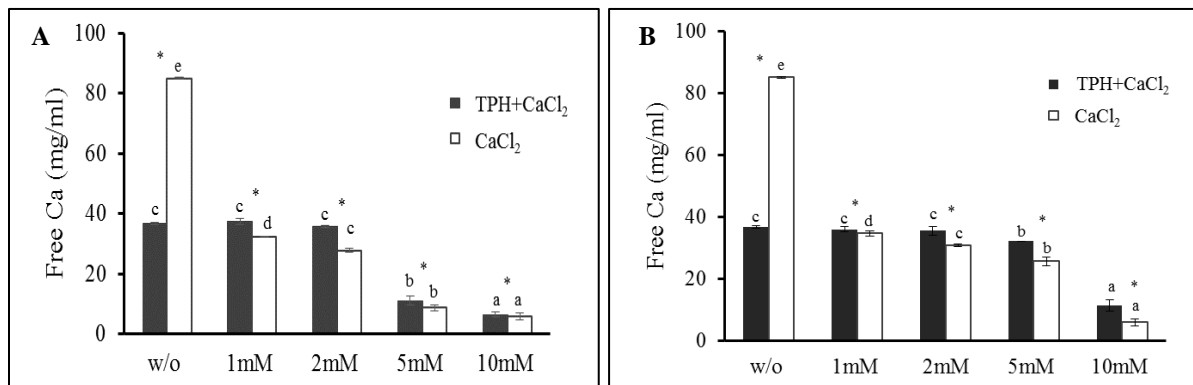


Figure 6. TPH reduced calcium precipitation by chelating agents. (A) Oxalic acid. (B) Phytic acid. Mean values with different letters were significantly different (p < 0.05).

TPH-calcium binding activity maintained in the simulated human digestion system

Food protein digestion occurs mostly in the stomach and duodenum. The primary digestive enzymes are pepsin secreted by the stomach and pancreatin – a collection of enzymes secreted by the pancreas. To evaluate the effect of these digestive enzymes on TPH-calcium-binding, TPH was subjected to in vitro pepsin-pancreatin digestion in a simulated human digestion system (Figure 7). The calcium-binding activities of TPH were not considerably different before and after simulated gastrointestinal digestion. This activity was reduced only by 5% at 3 h in pepsin digestion, whereas it was slightly increased at 2 h in pancreatin digestion (Figure 7A). This indicated that the calcium-binding activity of TPH was stable in this in vitro digestion model. Other bio-functional peptides or hydrolysates also showed similar gastrointestinal stability. The horse mackerel protein hydrolysate was stable for 5 h in a simulated two-stage gastrointestinal digestion [29].

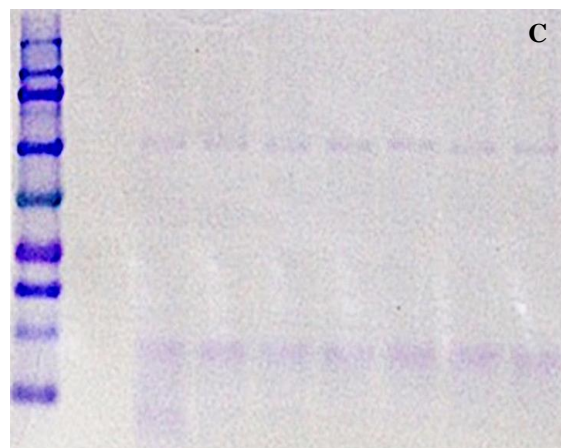
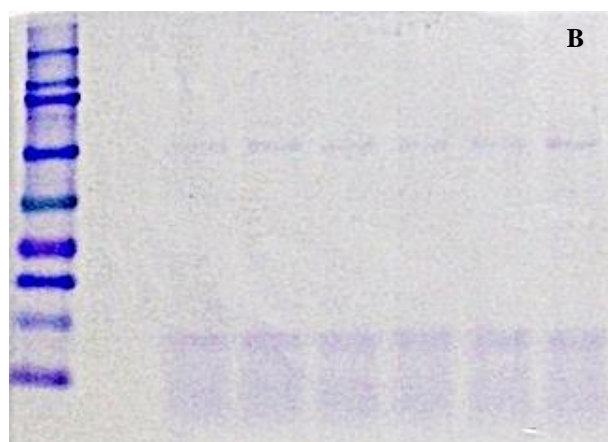
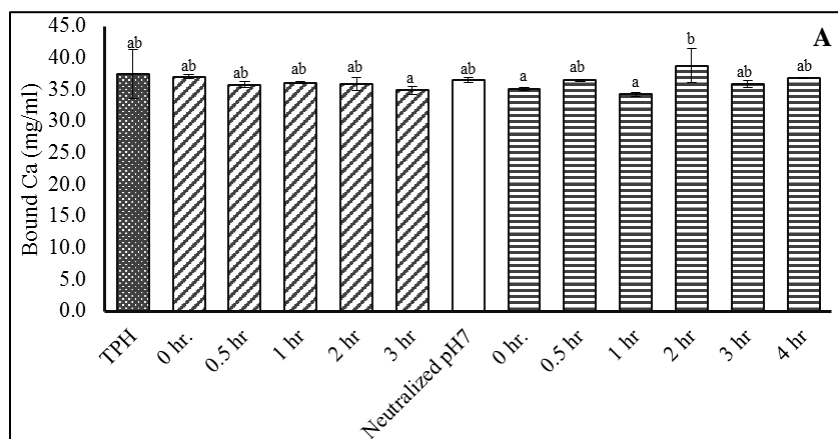


Figure 7. The stability of TPH-calcium-binding in the simulated gastrointestinal digestion system. (A) Time course of TPH-calcium-binding activities during sequential pepsin-pancreatin digestion. Mean values with different letters were significantly different ($p < 0.05$). Tricine SDS-PAGE analysis of the peptide patterns of TPH after digestion by (B) pepsin and (C) pancreatin.

The peptide distribution pattern of the hydrolysate was not affected by this digestion. The calcium-binding capacity of the hydrolysate did not change significantly either during the digestion. Another study showed that the in vitro pepsin-pancreatin digestion of milk and kefir resulted in a slight increase in their calcium-binding capacities [30]. This digestion made milk proteins relatively resistant to proteolytic degradation by kefir culture during fermentation.

The peptide patterns of TPH, however, were slightly different before and after simulated gastrointestinal digestion (Figures 7B-C). Before digestion, there were three major groups of peptides in the hydrolysate: bands at the 10 kDa, 15 kDa, and 50 kDa. After digestion, the amount of the peptides at 10 kDa band was significantly reduced. This suggested that these peptides were not responsible for calcium binding as the calcium-binding activities of TPH remains the same before and after digestion (Figure 7A).

To explore the potential of TPH as a functional food for promoting calcium absorption, the calcium binding of TPH was assessed for gastrointestinal stability and compatibility with food matrices. The calcium-binding activity of TPH will probably be maintained in vivo after its consumption because the binding was stable in the simulated gastrointestinal digestion system. Pepsin and pancreatin did not considerably change the calcium-binding activity although there were some changes in the peptide patterns of the hydrolysate. The calcium binding of TPH was also compatible with food matrices. Most food components tested including all saccharides, amino acids and vitamins showed positive or no effects on calcium-binding. This suggested that the calcium-binding activity of TPH will likely be preserved in the complex multi-component food matrices. However, some food components including fatty acids and a high concentration of iron reduced calcium-binding. This implicated that the calcium binding activity of TPH could be maximized if the food contains low levels of fatty acids and iron.

TPH enhances transepithelial calcium transport

Peptide binding to calcium has the potential to improve calcium uptake. One possible mechanism is the binding enhancing calcium across the intestinal epithelium. To test if TPH had such an effect, calcium transport was studied using an intestinal permeability cell model in which Caco-2 cells were cultured as monolayer used to predict calcium absorption across tight junctions. Caco-2 cells are human epithelial colorectal adenocarcinoma cells that are commonly used for studying the intestinal permeability of drugs and minerals [31, 32, 33]. Differentiated Caco-2 cells form tight junctions functionally similar to enterocytes lining the small intestine. The result found that TPH was not toxic to Caco-2 cells at a concentration of up to 1 mg/ml, the percentage of cell viability still above 80% at this concentration (Figure 8A). Because of its minimal effect on cell viability, the concentrations of TPH used in subsequent calcium transport experiments all referred to this concentration.

In the absence of TPH, calcium transport was time and concentration-dependent (Figure 8B). The transport of calcium was linear between 30 and 150 min at calcium concentrations of 2.5 and 5.0 mM but became non-linear at higher calcium concentrations (7.5 and 10.0 mM) (Figure 8B). The concentration dependence of calcium transport was non-linear for all time points tested (Figure 8B), which suggested the presence of a saturable transport component. In the presence of TPH, calcium transport was changed in its time dependence behavior in which the calcium transport became non-linear between 30 and 150 min (Figure 8C). However, TPH significantly enhanced calcium transport across the Caco-2 cell monolayer (Figure 8D). The permeability coefficient (Papp) of calcium transport in the presence of TPH was significantly higher than that in the absence of TPH. The highest permeability coefficient was observed with 1.0 mg/ml of TPH in which there was more than a two-fold increase in calcium transport. However, further increasing TPH concentration (to 2 mg/ml) reduced calcium transport. This result was concordance with the previous report from CPPs [34], one possible reason was reduced cell viability when TPH

concentration was higher than 1 mg/ml (Figure 8A). Nevertheless, our result demonstrated that TPH promoted calcium absorption in vitro cell permeability model.

TPH has the potential to enhance calcium bioavailability. This hypothesis was based on two findings from this study. We found that calcium precipitation by oxalate and phytate was significantly reduced in the presence of TPH. Oxalate is known to interfere with calcium absorption by forming insoluble salts of calcium. Phytate is a hexaphosphate of inositol and greatly inhibits calcium uptake [35]. This suggested that TPH could potentially enhance calcium absorption in high-oxalate or high-phytate foods.

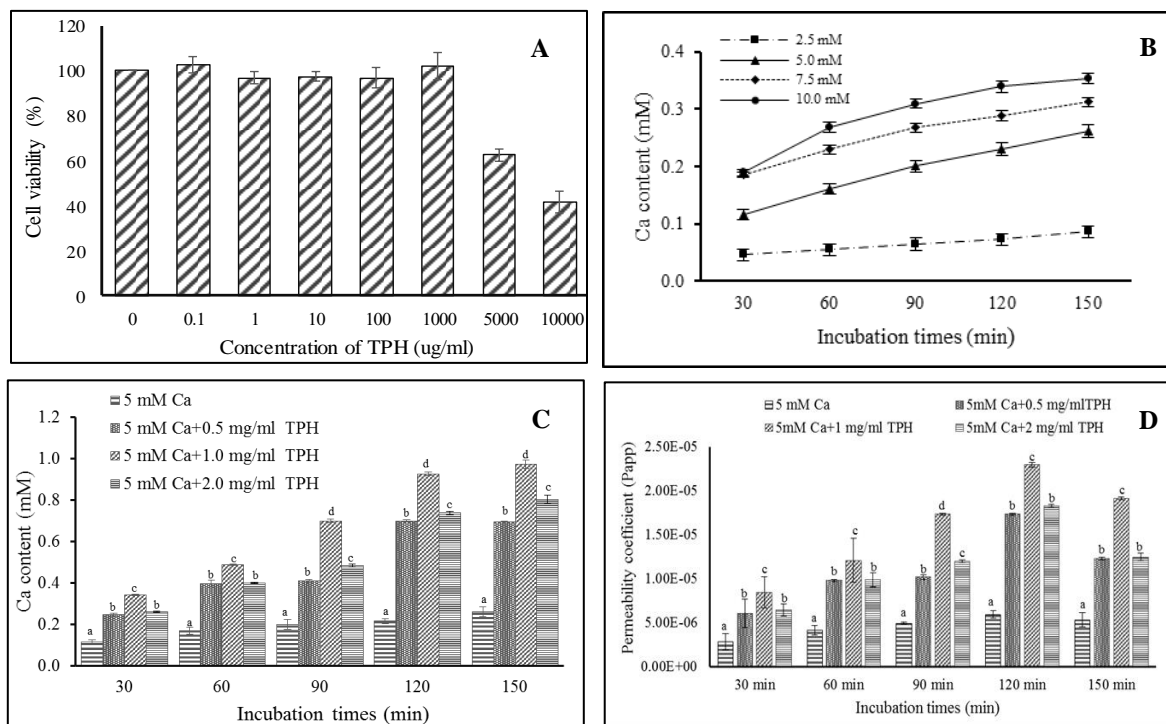


Figure 8. TPH enhanced calcium transport across the Caco-2 cell monolayer. (A) Effects of TPH on Caco-2 cell viability. Calcium transport in the absence of TPH (B). Calcium transport in the presence of TPH (C) and as a function of time. TPH increased the permeability coefficients of calcium transport (D). Mean values with different letters were significantly different ($p < 0.05$).

The second finding that supported the health-promoting potential of TPH was TPH enhancing transepithelial calcium transport in the Caco-2 cell permeability model. Caco-2 cells are widely used as an in vitro model of the human small intestinal mucosa [36]. Because of differentiated Caco-2 cells morphologically and functionally resembling the intestinal barrier, they have been extensively used for intestinal transport studies [17]. This cell model mimics processes including transcellular transport, paracellular transport, and some aspects of efflux and active transport [37]. While the exact mechanisms of TPH promoting calcium transport need further investigation, both paracellular and transcellular transports could be affected by the calcium-binding peptides in TPH. Peptides can serve as a carrier for paracellular transport of chemicals across the tight junction [38]. They can also directly target proteins of the apical junctional complex causing the opening of the tight junction [39]. However, recent studies showed that casein phosphopeptides (CPPs) were likely to influence the transcellular pathway of intestinal calcium absorption rather than the paracellular pathway [40]. Enhanced calcium transport in Caco-2 cells was due to CPPs affecting

the transcellular pathway by upregulating the expression of TRPV6. Nevertheless, the ability of TPH to bind calcium, promote transepithelial calcium transport and prevent calcium precipitation by the chelating agents warranted future investigation of its effects on calcium bioavailability and homeostasis.

CONCLUSION

Tilapia protein hydrolysate (TPH) produced by alcalase hydrolysis of Nile tilapia was shown calcium-binding activity. However, such an activity should probably be maintained *in vivo* after its consumption because the binding was stable in the simulated gastrointestinal digestion system. The binding was also compatible with food matrices. Most food components tested including all saccharides, amino acids, and vitamins showed positive or no effects on calcium binding. However, some food components including fatty acids and a high concentration of iron reduced calcium binding. This implicated that the calcium-binding activity of TPH could be maximized if the food contains low levels of fatty acids and iron. Also, TPH has the potential to enhance calcium bioavailability. Calcium precipitation by oxalate and phytate was significantly reduced in the presence of TPH and that TPH enhanced transepithelial calcium transport in the Caco-2 cell permeability model. These together support a health-promoting potential of TPH.

List of Abbreviations: CPPs, casein phosphopeptides; TPH, tilapia protein hydrolysate; ATCC, The American Type Cell Collection; TEER, transepithelial electrical resistance.

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