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Desalted Duck Egg White Peptides: Promotion of Calcium Uptake and Structure Characterization

Tao Hou,[†,‡] Chi Wang,[†,‡] Zhili Ma,[†,‡] Wen Shi,[†,‡] Lui Weiwei,[†,‡] and Hui He*[,†,‡]

[†]College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

[‡]Key Laboratory of Environment Correlative Dietology (Huazhong Agricultural University), Ministry of Education, Wuhan 43000, China

ABSTRACT: The effects of desalted duck egg white peptides (DPs) on calcium absorption were investigated in three models: Caco-2 cell monolayer model, Caco-2 cell population model, and everted intestinal sac model. DPs were found to enhance calcium transport and may do so by acting as calcium carriers and interacting with the cell membrane to open a special Ca^{2+} channel, whereas the paracellular pathway may make only a minor contribution. Structure characterization demonstrated the important roles of seven crucial peptides, such as VSEE and LYAEE, in binding calcium and promoting calcium uptake. Three synthetic peptides (VHSS, VSEE, and VHS(p)S(p)) potently induced calcium transport in Caco-2 monolayers, with VHS(p)S(p) being the most effective. This research expands the understanding of the mechanism of cellular calcium uptake by DPs as well as highlights an opportunity for recycling an otherwise discarded processing byproduct.

KEYWORDS: *desalted duck egg white peptides (DPs), calcium uptake, Caco-2 cells, everted intestinal sac model, structure characterization*

INTRODUCTION

Calcium is one of the most abundant inorganic elements in the human body and has many important physiological roles; thus, it is a key player in human health and disease. Besides being a major component of bones and teeth, calcium also helps to maintain the permeability of blood capillaries and cell membranes, acts as a messenger in regulating cell division and apoptosis, and participates in various other physiological activities such as muscle contraction. Therefore, finding a substance that can efficiently bind calcium as well as promote its absorption by the intestine would be valuable.

Many calcium supplements are reported to be able to promote calcium uptake, such as chicken eggshell matrix proteins,[1] soybean protein,[2] whey protein,[3] and bovine serum protein hydrolysates.[4] Casein phosphopeptides (CPPs) are considered mineral carriers with a potential role in improving calcium absorption.[5,6] CPPs and phosvitin phosphopeptides (PPPs) are universally accepted calcium-promoting peptides. CPPs and PPPs increase intestinal calcium absorption via the phosphate residues of serine, which chelate with calcium to form soluble and stable complexes.[7] The amino acid composition and the sequence of the peptides determines their effects on calcium absorption.[8] Some calcium-binding soybean protein hydrolysates have been identified, and the Glu-, Asp-, and Pro-containing structures are considered to play a crucial role in binding with calcium.[9]

Concerning the mechanism of calcium absorption, a previous study using the Caco-2 cell monolayer model found that the calcium transcellular pathway represents a small percentage of total calcium transepithelial transport, whereas a major contribution of the paracellular pathway could not be substantiated.[1] However, other studies are controversial. Some researchers have claimed that CPP enhanced calcium uptake from extracellular medium by a direct action on the plasma membrane without affecting the intracellular calcium stores.[10] Another study pointed out that the interaction of the peptides with the TRPV6 calcium channel or with voltage-operated L-type calcium channel may trigger calcium entry into the cells, resulting in increased calcium uptake.[11,12] Taken together, these studies suggest the existence of multiple mechanisms of calcium uptake mediated by peptides with different molecular structures.

Desalted duck egg white peptides (DPs) are the processing byproduct hydrolysates of salted eggs and are readily available because large amounts of salted egg white are discarded due to the 7−10% sodium chloride content. Our previous study has demonstrated that desalted duck egg white peptides, phosphorylated DPs, and DPs−calcium complexes were all calcium absorption promoters and could enhance bone tissues and mechanical properties in rapidly growing rats.[13] However, the biochemical mechanism remains to be elucidated, and little information is available about the utilization of DPs as calcium-binding peptides. With this in mind, the purposes of the present study were to evaluate the effect of DPs on calcium uptake in three models and to clarify the possible mechanism underlying calcium absorption by DPs. Additionally, the structure of DPs was also investigated.

MATERIALS AND METHODS

Materials and Reagents. Salted duck egg white with a sodium chloride content of 7% and a protein content of 10% was donated by Hubei Shendan Healthy Food Co., Ltd. (Wuhan, China). Cell culture media and fetal bovine serum were purchased from GIBCO (Grand Island, NY, USA). Fluo-3 acetoxymethl ester (Fluo-3/AM) and Pluronic

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F-127 were purchased from Biotium Inc. (Hayward, CA, USA). HPLC-grade acetonitrile was purchased from Fisher Chemical (Bridgewater, NJ, USA), and HPLC-grade water was prepared using a Milli-Q system (Millipore Ibérica, Madrid, Spain). Trifluoroacetic acid (TFA) of HPLC grade was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals employed were of analytical reagent grade, and the water used was double-deionized.

Preparation of Desalted Duck Egg White Peptides (DPs). DPs with molecular weight of <5 kDa were prepared from salted duck egg white as described in our previous work.[13] Salted duck egg white was desalted by electrodialysis equipment, and then the desalted duck egg white was denatured for 30 min in boiling water and cooled to 50 °C. The pH was adjusted to 6.5 prior to the addition of protamex (E:S = 1:25) and maintained at 6.5. After 3.5 h, the hydrolyzed solution was bathed in boiling water for 10 min to inactivate the enzyme. Finally, the mixture was centrifuged at 3000*g* for 10 min, and the supernatant was filtered through a hollow fiber membrane with a molecular weight cutoff of 5 kDa (PLCC, Millipore, Billerica, MA, USA). The filtrate was lyophilized and thereafter referred to as DPs.

Establishment of Caco-2 Cell Monolayers. The Caco-2 cells were used as a model of the intestinal epithelium, and this cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were routinely grown in 75 cm^2 plastic flasks (Greiner Bio-One GmbH, Germany) in MEM medium (GIBCO) with 15% fetal bovine serum, supplemented with 1% nonessential amino acids (GIBCO) in the presence of 100 u/mL streptomycin and 100 u/mL penicillin. The cells were then incubated at 37 °C with 5% CO_2. Cell passage numbers of 30−50 were used in this experiment. The culture medium was changed every 2 days until the flasks reached 90% confluence. Cell viability was assessed by the MTT test, and cell morphology was examined through an optical microscope.

At 90% confluence, the Caco-2 cells were detached with trypsin−EDTA treatment (final concentration of 0.005 g/L), followed by seeding of the cells on 12-well millicell cell culture inserts (0.4 μm pore size, 12 mm diameter, Millipore Corp., Billerica, MA, USA) with a cell density of 1.2×10^5 cells/mL and 21 days of incubation. The volume of culture medium was 1.95 mL in the basolateral side and 0.4 mL in the apical side. Transepithelial electric resistance (TEER) values were checked every 2 days by using a Millicell-ERS system (Millipore) to evaluate the tight junction permeability of the Caco-2 cell monolayers.

Calcium Transport Studies. Caco-2 cell monolayers with TEERs exceeding 500 $\Omega \cdot cm^{-2}$ were used for the calcium transport experiments. The monolayers were set in a 12-well plate and washed twice with warm (37 °C) balanced salt solution without calcium and magnesium (HBS) (125 mM NaCl, 4 mM KCl, 4 mM L-Glu, 10 mM glucose, 30 mM HEPES, adjusted to pH 7.4) and incubated at 37 °C for 20 min. The wells were then transferred to a new cluster plate containing 1.95 mL of HBS buffer. Different concentrations of calcium solution (0−20 mM, 0.4 mL) were added to the apical side of the inset, and the calcium transport rates across Caco-2 cell monolayers were measured every hour for 3 h. After the optimum conditions were determined, DPs of different concentrations (0−8 mg/mL) were added to the apical side with the optimum calcium solution. After incubation, samples were collected from the basolateral side for further analysis. The TEERs were monitored before and after the addition of the calcium and DP samples. The calcium contents were measured with an atomic absorption spectrophotometer (AA6300C, Shimadzu, Kyoto, Japan).

Measurement of Ca^{2+} in Caco-2 Cell Population Model. At 90% confluence, the Caco-2 cells were detached with trypsin−EDTA treatment and then washed several times with HBS. About 4×10^6 cells were loaded in HBS with 5 μmol/L Fluo-3/AM and 2.5 μmol/L Pluronic F-127 for 30 min at 37 °C. The loaded cell suspension was rinsed thoroughly two times with calcium-free HBS and then divided into eight portions, each containing 0.5×10^6 cells. Each aliquot was suspended in 1 mL of HBS containing different calcium concentrations (2−6 mM) and then transferred to a cuvette and placed in a Hitachi F-4600 spectrofluorometer (Hitachi, Japan). The cells were stirred continuously during the experiment, and fluorescence excitation/emission wavelengths were set at 488/525 nm for Fluo-3-loaded cells. Then different concentrations of DPs were added into the cuvette through the injection port. To maintain cell viability, the duration of the whole experiment never exceeded 20 min. The results in each case were expressed as Ca^{2+} peak of fluorescence intensity units from baseline.

Everted Rat Intestine Permeation. Wistar male rats in the weight range of 250−300 g were obtained from Hubei Laboratory Animal Research Center and kept under standard environmental conditions. The animals were handled in accordance with the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals and the Chinese standards (GB 14925-2001). Food was removed about 18 h prior to trial, but water was allowed ad libitum before sacrifice. The entire small intestine was immediately collected via a midline incision of the abdomen and placed into the buffer solution (136 mM NaCl, 8.17 mM KCl, 1.0 mM $MgCl_2$, 11.1 mM glucose, 20 mM HEPES, adjusted to pH 7.4) at 4 °C. The first 15 cm of duodenum was discarded, and the intestine was washed thoroughly with the buffer. The surface liquid was sopped up by the filter paper, and then the intestine was everted. Each everted intestinal segment was about 6 cm in length, and 4 mL of buffer solution (drug free) was then placed into the serosal compartment. The two distal ends were ligatured, and the entire everted intestine segment was placed into the flasks containing 100 mL of buffer with various concentrations of DPs and calcium at 37 °C. Meanwhile, 95% O_2 was constantly bubbled through the buffer. After 120 min of incubation, the serosal solution was collected and the transported calcium was measured with an atomic absorption spectrophotometer (AA6300C, Shimadzu, Kyoto, Japan).

HPLC Analysis Instrumentation. The HPLC analysis was performed on a Waters e2695 HPLC-DAD system (Waters, Milford, MA, USA), equipped with a diode array detector, a vacuum degasser, an autosampler, a binary pump, and a column compartment. DPs (<5 kDa) at a concentration of 10 mg/mL were analyzed on a ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μm, Agilent Co., Santa Clara, CA, USA) at a flow rate of 1 mL/min, with the temperature of the column maintained at 30 °C. A binary gradient elution system, comprising water (with 0.1% TFA, A) and acetonitrile (with 0.1% TFA, B), was applied as follows: 0.0−20.0 min, 5−15% B; 20.0−35.0 min, 15−20% B; 35.0−40.0 min, 20−30% B; 40.0−50.0 min, 30−40% B. The volume of injection was 10 μL, and the DAD detector was set at 220 nm for analysis.

HPLC-ESI-MS/MS Analysis Instrumentation. For HPLC-ESI-MS/MS analysis, the HPLC system described above was replaced by an Agilent1100 series HPLC-ESI-MS system (Agilent Technologies) but with the same column and gradient elution as in the HPLC analysis. Mass spectrometry conditions were set as follows: ESI^+ ion source; drying gas temperature, 325 °C; nebulizer, 40.00 psi; dry gas flow rate, 10.0 L/min; capillary voltage, 3500 V; scan spectra from m/z 100 to 1000.

The sequences were identified using LCMSD-Trap Data Analysis software and confirmed by searching the online MS database on the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mspattern).

Isothermal Titration Calorimetry (ITC). ITC was undertaken with an ITC200 calorimeter (MicroCal, Northampton, MA, USA). The titrant was 10 mM $CaCl_2$, and the sample cell contained 0.5 mM peptides or 0.4 mM EDTA dissolved in double-distilled water (pH 7.8). The titration comprised 20 × 2 μL injections at 25 °C. Data were analyzed with the Origin 5.0 software package (ITC Data Analysis in Origin, MAU130010 REV F-4, MicroCal). The binding constants K, ΔH, and ΔS were calculated.

Statistical Analysis. The sample treatments described in this experiment were conducted in triplicate, and the data were presented as mean values ± SD. Data were calculated using one-way ANOVA of SPSS 18.0 followed by Duncan's multiple-range test. Differences were considered significant at P values <0.05.

RESULTS AND DISCUSSION

Permeability of Caco-2 Cell Monolayers. Native epithelial tissues are traditional subjects for the research of transport processes, but these tissues are complex and contain many different cell types. Caco-2 cells are human intestinal

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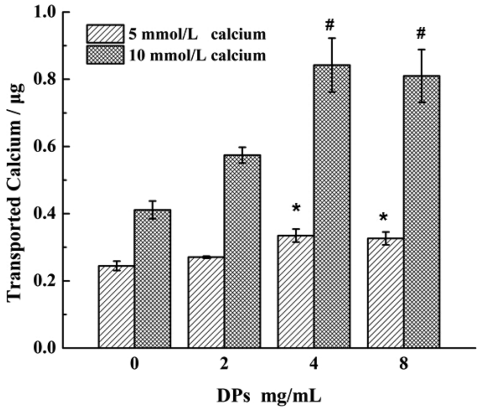

Figure 1. Effects of DPs on calcium transport across Caco-2 monolayers. DPs (0−8 mg/mL) were added to the apical side with calcium solution (5 or 10 mM, 0.4 mL) and then incubated for 120 min. Mean values with different letters are significantly different at $P < 0.05$.

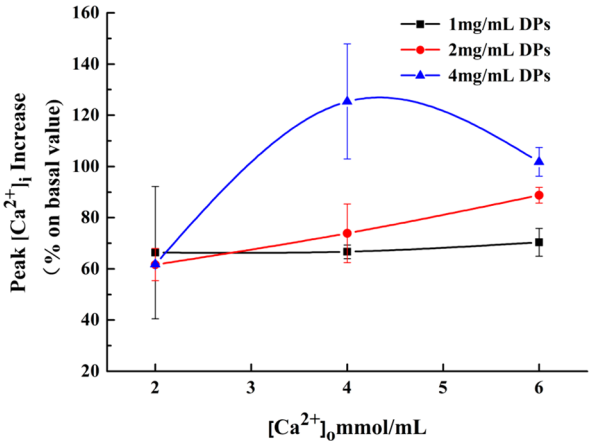

Figure 2. Calcium uptake by Caco-2 cells as influenced by different concentrations of DPs (1−4 mg/mL) and extracellular Ca^{2+} (0−4 mM). Intracellular calcium concentrations are expressed as an increase in fluorescence intensity compared to baseline, which is the original fluorescence intensity before the addition of DPs to the aliquot containing 0.5×10^6 cells.

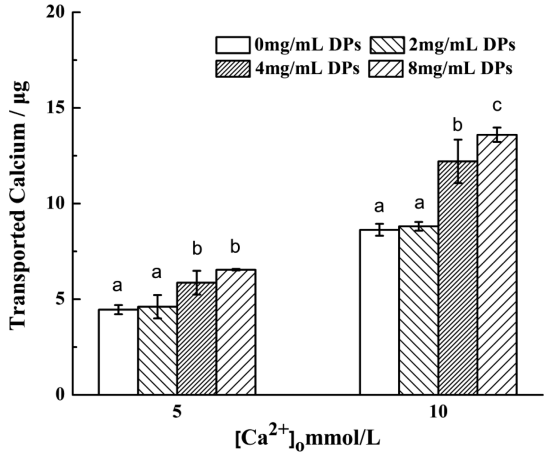

Figure 3. Effects of DPs on calcium transport across everted intestinal sac. DPs (0−8 mg/mL) were added to the mucosal solutions containing 5 or 10 mM Ca^{2+} and then incubated for 120 min. Mean values with different letters are significantly different at $P < 0.05$.

Table 1. Amino Acid Composition (g/100 g Peptides) of DPs (M_w < 5 kDa)

amino acid	DPs (<5 kDa)	amino acid	DPs (<5 kDa)
Asx[a] (D + N)	7.14	Ile (I)	3.3
Thr (T)	5.19	Leu (L)	7.17
Ser (S)	7.44	Tyr (Y)	3
Glx[b] (E + Q)	12.14	Phe (F)	6.17
Gly (G)	2.84	Lys (K)	4.5
Ala (A)	3.95	His (H)	1.54
Cys (C)	1.16	Arg (R)	3.52
Val (V)	4.97	Pro (P)	2.11
Met (M)	5.1	total amino acid	81.24

[a]Asx = Asp + Asn (D + N). [b]Glx = Glu + Gln (E + Q).

adenocarcinoma cells exhibiting enterocyte-like biochemical and morphological characteristics, and they have been reported to be an excellent in vitro model of human enterocytes for absorption studies involving minerals,[14] polyphenols,[15] proteins, and peptides.[16] In the present study, Caco-2 monolayers were used to evaluate the effect of DPs on calcium transport.

After cells had been seeded on 12-well millicell cell culture inserts, different concentrations of calcium were added to the apical compartment and the calcium on the basolateral side was measured. When 5 or 10 mM Ca^{2+} was added, no significant change in calcium transport was observed. When the concentration was increased to 20 mM, the calcium transport showed a 2-fold increase compared to control. As the total Ca^{2+} concentrations in the intestinal lumen may reach 3−4 mM in rats and 7−8 mM in humans after a proper meal,[17] 5 and 10 mM were chosen as suitable Ca^{2+} concentrations for further experiments. The effect of incubation time on calcium transport was then evaluated and found to increase with the extension of incubation time. On the basis of the retention time of food in the intestine (about 120−180 min) and cell viability, an incubation time of 120 min was determined to be the optimum duration for further experiments (data not shown).

Effect of DPs on Calcium Transport in Caco-2 Monolayers. Cell viability was evaluated to be >90% after treatment with different DPs concentrations (0−8 mg/mL). Figure 1 shows the effect of 0−8 mg/mL DPs on calcium transport with 5 and 10 mM Ca^{2+} in Caco-2 cell monolayers. In the presence of 4 and 8 mg/mL DPs, the calcium transport showed increases of 36.77 and 33.33% over that of the control group at the concentration of 5 mM Ca^{2+}. Increasing the concentration of Ca^{2+} to 10 mM increased the calcium transport by 104.88% (4 mg/mL DPs) and 97.01% (8 mg/mL DPs) versus the control group. For a given calcium concentration, the increase in Ca^{2+} transport was roughly equal whether 4 or 8 mg/mL DPs were used, implying that the influence of DPs on the transport process was saturable.

Drugs may be transported across the intestinal epithelium by one or more of four different routes: passive transcellular and paracellular routes, carrier-mediated route, and transcytosis.[18] Because the surface area of the brush border membranes is about 1000-fold larger than the paracellular surface area,[19] drugs can be assumed to be transported mainly by the transcellular route. The TEER value is a highly sensitive measure of membrane permeability, and its variation largely reflects the function of tight junctions that gate the entrance to the paracellular pathway. TEER values showed no significant difference after 120 min of incubation with different concentrations of DPs and calcium. Thus, our results from the Caco-2 cell monolayer experiment

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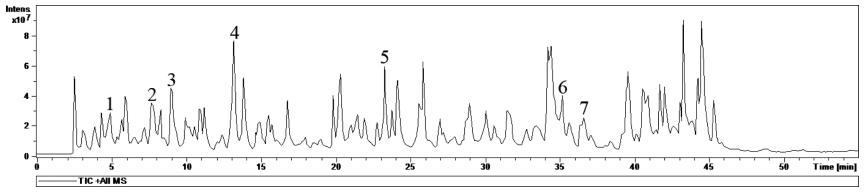

Figure 4. Total ion current (TIC) chromatogram of DPs (<5 kDa). Seven peaks of DPs with M_w < 5 kDa in the TIC chromatogram are numbered.

Table 2. Structure Summary of DPs (<5 kDa) Using HPLC-ESI-MS, MS/MS[a]

peak	retention time (min)	peptide $[M + H]^+$	sequence	protein name
1	5.0	383.2037	VHQ	ovostatin
2	7.7	463.2035	VSEE	ovalbumin
3	8.8	375.1874	INE	ovalbumin
4	13.2	487.3239	ILKN	lysozyme precursor
5	23.3	624.2875	LYAEE	ovalbumin
6	35.1	519.2562	INSW	ovalbumin
				ovalbumin-related protein Y
7	36.4	495.2813	LSYL	ovoglobulin

[a]The primary structures of the DPs (<5 kDa) were determined and validated by searching three mass spectrometry databases of SwissProt.2013.6.27, NCBInr.2013.6.17, and UniProtKB.2013.6.17 with the Protein Prospector engine.

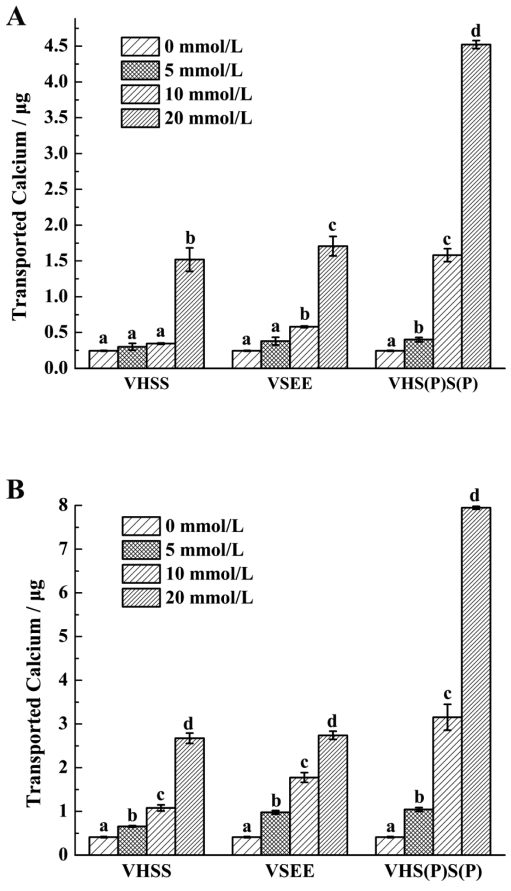

Figure 5. Effects of three pivotal peptides (VHS(p)S(p), VSEE, and VHSS) on calcium transport across Caco-2 monolayers. Peptides (0−20 mM) were added to the apical side with calcium solution (5 mM (A) and 10 mM (B), 0.4 mL) and then incubated for 120 min. Mean values with different letters are significantly different at $P < 0.05$.

confirmed the hypothesis that the paracellular pathway was a minor contributor to calcium transport by DPs. This conclusion is in contrast to the traditional theory holding the paracellular pathway as the major route of calcium transport across Caco-2 cell monolayers.[20] Various mechanisms have been reported to contribute to the absorption of calcium from the intestine, and the paracellular way has been confirmed for many bioactive substances such as vitamin D[21] and CPP.[22] However, the challenge to clearly describe calcium transport at the cellular level remains. Now, our current results provide a new insight into the DPs-mediated calcium transport pathway.

Effect of DPs on Calcium Uptake in Caco-2 Population Model. The effect of DPs (1−4 mg/mL) on the intracellular free calcium concentration, $[Ca^{2+}]_i$, was found to be concentration-dependent in the cell population model with 4 and 6 mM Ca^{2+} (Figure 2). This result is consistent with published studies of CPPs[8] and soluble soybean protein hydrolysate−calcium complex,[2] which may act as calcium carriers and interact with the plasma membrane to transport calcium to the cytosol. This function has been reported to be related to the β-pleated sheet structure of the peptides, which can interact with the plasma membrane to form unregulated Ca^{2+} channels.[23] The β-pleated sheet structure was also found in calreticulin, which is involved in calcium binding in the lumen of the endoplasmic reticulum.[24]

In addition, the optimal DPs/Ca^{2+} ratio for the enhancement of Ca^{2+} uptake was observed to be 4 mM Ca^{2+}/4 mg/mL DPs, indicating the importance of a suitable DPs/Ca^{2+} ratio for promoting calcium absorption. A similar finding was also reported for CPPs in both in vitro cell model[17] and animal models.[25]

Effect of DPs on Calcium Transport in Everted Intestinal Sac Model. Figure 3 shows the amount (μg) of Ca^{2+} absorbed across the everted intestinal sac (6 cm) with mucosal solutions containing 5 or 10 mM Ca^{2+} and 0−8 mg/mL DPs. Using 5 mM Ca^{2+}, calcium transport increased by 31.71% over control with 4 mg/mL DPs and by 46.95% with 8 mg/mL DPs. Similar to the findings in the Caco-2 cell monolayers, the extent of Ca^{2+} transport increase was similar whether 8 or 4 mg/mL DPs was used. At the 10 mM Ca^{2+} concentration, calcium transport exhibited a significant increase over control in the presence of 4 mg/mL DPs (41.45%, $P < 0.05$) and 8 mg/mL DPs (57.59%), but not 2 mg/mL DPs. These results indicate the various Ca^{2+} concentrations are a main driver behind the increase in calcium transport and thus may influence the involvement of the paracellular pathway. Thus, we conclude the paracellular pathway may not be the main transport route in the presence of 5 mM Ca^{2+} concentration. However, when the external concentration of Ca^{2+} was increased to 10 mM, the entrance of the paracellular pathway was opened.

Despite being an in vitro model, the everted intestinal sac model has the advantage of being similar to in vivo intestinal absorption, which has been used in the study of calcium absorption by CPPs.[26] A CPP/Ca ratio of 15 was found to have a significant effect on mineral absorption, and the positive effect of

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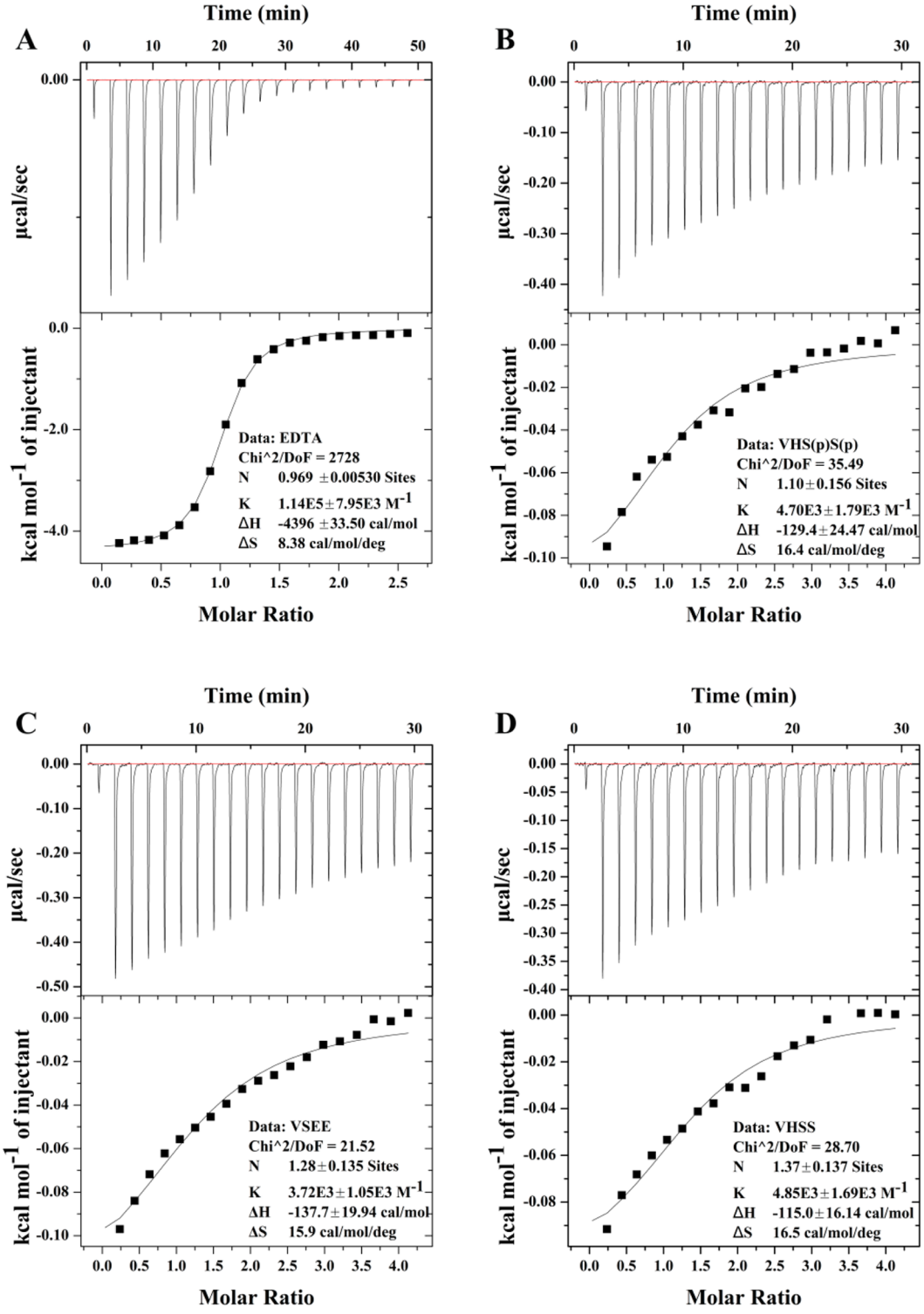

Figure 6. Thermograms and binding isotherms from ITC analysis of the interactions between (A) EDTA and (B) VHS(p)S(p), (C) VSEE, and (D) VHSS at 30 °C.

CPPs on passive Ca absorption seems to depend on the relative amounts of both species in the intestinal lumen. In this study, from both the Caco-2 cell monolayer and everted intestinal sac models, the same conclusion can be drawn: in the presence of DPs, the paracellular pathway is not the main route of calcium absorption.

Identification of High Bioactivity of DPs. The ability of DPs to promote calcium absorption was affected greatly by the amino acid composition of the peptides. The most abundant amino acids in the DPs (<5 kDa) were Glx (Glu + Gln) and Asx (Asp + Asn), accounting for 12.14 and 7.14% of the mass of the peptide, respectively. The contents of Ser, Leu, Phe, Thr, and Met were each also >5% (Table 1). Glu and Ser amino acid residues were also present in the calcium-binding peptides derived from cheese whey protein hydrolysates,[27] porcine blood plasma protein hydrolysates,[28] and Hoki protein hydrolysates.[29]

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The carboxyl in some acidic amino acids, the δ-N of the imidazole ring of histidine (His), and N in amidogen of the side chain were the optimal binding sites of Ca^{2+}, and these three amino acids accounted for 35.50% of the total amino acids, which was close to the 37.64% found in soybean peptides.[30]

According to the retention time, seven peaks from DPs with M_w < 5 kDa in the total ion current (TIC) chromatogram were numbered (Figure 4), and the amino acid sequences of the seven peaks were identified as Val-Ser-Glu-Glu (VSEE), Leu-Tyr-Ala-Glu-Glu (LYAEE), Ile-Asn-Glu (INE), Leu-Ser-Tyr-Leu (LSYL), Leu-Asn-Ser-Trp (LNSW), Val-His-Gln (VHQ), and Ile-Leu-Lys-Asn (ILKN) (Table 2). The peptides possess the characteristics of high contents of E, L, N, and S, which is consistent with the result of amino acid composition of DPs. In our previous work, we described a DPs−calcium chelate found using Fourier transform infrared spectroscopy and fluorescence spectroscopy of DPs.[31] The results indicated that the carboxyl groups in some acidic amino acids were the optimal binding sites for Ca^{2+}. The calcium ions caused peptide folding and led to the formation of a DP−calcium chelate. Interestingly, a repeating Glu sequence was found in the C-terminal of VSEE and LYAEE, providing many potential binding sites (the carboxyl groups) for calcium. "Glu-Glu" was also found in the characteristic "acidic motif" Ser(P)-Ser(P)-Ser(P)-Glu-Glu in CPPs.[32] Ser was also found to exist in the peptides LSYL and LNSW, the phosphorylation of which may improve calcium binding, as it has been reported that the phosphorylation of peptides derived from isolated soybean protein could enhance the absorption of calcium in Caco-2 cells.[33] The existence of His in VHQ may contribute considerably to binding calcium due to the δ-N of the imidazole ring. One calcium-binding peptide, Thr-Cys-His, has been identified from a shrimp-processing byproduct hydrolysate, and its high calcium-binding activity may be due to the presence of a His residue.[34] N in amidogen of a side chain of Lys in ILKN was a binding site with calcium, and Lys occupied 4.5% of the mass in DPs (<5 kDa). Lys also been identified in some calcium-binding peptides from tilapia scale protein hydrolysate and bovine serum protein hydrolysates.[4] From the results of those studies, it can be deduced that Glu, Asp, His, and Ser residues may play a crucial role in the chelating of DPs with calcium.

Effect of Three Pivotal Peptides on Calcium Transport in Caco-2 Monolayers. VSEE and VHSS (which had been identified in our laboratory) and phosphorylated VHSS (VHS(p)S(p)) were synthesized by HeFei GuoTai Biology Co., Ltd. (Hefei, China). Figure 5 shows the effect of these three peptides (0−20 mM) on calcium transport, using 5 and 10 mM Ca^{2+}, in Caco-2 cell monolayers. In the presence of 5 mM Ca^{2+}, groups treated with the lower dosage of VHSS and VSEE did not show any significant increase in calcium transport over the control group. As the concentration of peptides was increased to the highest dose (20 mM), the calcium transport increased by >200%. At all doses tested, calcium transport facilitated by VHS(p)S(p) was always higher than that of the other two peptides. In the presence of 10 mM Ca^{2+}, each of the doses tested of VHSS, VSEE, and VHS(p)S(p) stimulated calcium transport.

Figure 6 shows the ITC curves of Ca^{2+} titrated into peptide solutions, as well as the thermodynamic parameters for interaction of peptides with calcium including the stoichiometry (n), association constant (K), change in enthalpy (H), and change in entropy (S). The binding association constants were similar for all peptides, but significantly lower than those for EDTA, which indicates that these three peptides could release calcium easily. The stoichiometry (n) demonstrated a significant difference in how the calcium is bound to peptides. VHS(p)S(p) provides seven binding sites, including an oxygen atom from a hydroxyl in the C-terminal carboxyl, a δ-N atom in the imidazole ring of histidine (His), a N atom of amidogen in the N-terminal, and four oxygen atoms in the phosphate group. Therefore, a small amount of VHS(p)S(p) can bind many calcium ions, which supports the results seen in the Caco-2 monolayers, indicating that the binding activity of peptides is necessary in the process of calcium transport in this cell model. VHSS and VSEE contained three and four functional chelating sites, respectively, so they also could act as calcium-binding peptides. In addition, the three peptides were all detected by HPLC in the basolateral samples, indicating that all three peptides participated in calcium transport, most likely as carriers (data not shown).

On the basis of the mechanisms of calcium uptake in the three models, the amino acid composition, and the sequences of the peptides, the contribution of DPs to calcium absorption may be attributed to two factors: (1) the structure of DPs can ensure high bioactivity is maintained, in order for calcium to interact with the membrane and open the specific calcium channels; and (2) the most suitable DPs structures will not only bind calcium for transport but also release the ion easily.

In summary, the present study provides evidence from three experimental models that DPs (<5 kDa) have a significant effect on calcium absorption. Our results suggest that rather than relying on the paracellular pathway, DPs may facilitate calcium transport by acting as calcium carriers and interacting with the cell membrane to open a special Ca^{2+} channel. Furthermore, our findings shed light on the relationship between amino acid composition and bioactivity. Glu, His, Lys, and Ser in DPs may play crucial roles in the chelation of DPs with calcium, and some peptides containing pivotal amino acid sequences, such as VSEE, have high calcium-binding capability. Future work should focus on the relationship between the bioactivity and structure of DPs to help identify a peptide with the capability of not only binding desired quantities of calcium but also interacting with the plasma membrane to enhance the entrance of Ca^{2+} into cells.

AUTHOR INFORMATION

Corresponding Author

*(H.H.) Mail: College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China. Phone: 011-86-27-87280346. Fax: 011-86-27-87282966. E-mail: hehui@mail.hzau.edu.cn.

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Notes

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