Chicken Eggshell Matrix Proteins Enhance Calcium Transport in the Human Intestinal Epithelial Cells, Caco-2

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Chicken eggshell powder has been proposed as an attractive source of calcium for human health to increase bone mineral density in an elderly population with osteoporosis. However, factors affecting calcium transport of eggshell calcium have not yet been evaluated. Chicken eggshell contains about 1.0% (w/w) matrix proteins in addition to a major form of calcium carbonate (95%, w/w). In this study, we found that soluble eggshell matrix proteins remarkably enhance calcium transport using in vitro Caco-2 cell monolayers grown on a permeable support. The total calcium transport across Caco-2 monolayers showed an increase of 64% in the presence of 100 μg/well soluble eggshell matrix proteins. The active enhancer with a molecular mass of 21 kDa was isolated by reversed phase high-performance liquid chromatography and did not correspond to any previously identified protein. The N-terminal sequence was determined to be Met-Ala-Val-Pro-Gln-Thr-Met-Val-Gln. The possible mechanisms of eggshell matrix protein-mediated increase in calcium transport and the potential significance of eggshell calcium as a nutraceutical are discussed.

KEYWORDS: Eggshell; soluble matrix proteins; calcium transport; Caco-2; bioavailability; nutraceutical; reversed phase HPLC

INTRODUCTION

Calcium is known as the most abundant inorganic element in the human body, accounting for 1.5−2.2% of the body weight. The majority of it (about 99%) is present in bone. The primary function of calcium is to provide structural integrity and strength to both trabecular bones, such as the vertebrate, and cortical bones. Calcium is also necessary for the integrity of cell membranes and is essential for maintaining normal function in muscle, nerve, and secretary organs (*1*, *2*). For this reason, the calcium level in the extracellular compartment is kept within very narrow limits by the endocrine system concerned with calcium homostastasis. Regulating blood calcium levels is a dynamic and precise process, which ranges from 9 to 11 mg/dL. Calcium flux in the body is also dynamic with the dietary calcium intake balancing the daily loss of approximately 1000 mg. Bones are consistently renewing themselves (*1*). The level of the recommended dietary allowances for calcium is suggested to be as much as 1500 mg/day (*3*). There are two principal types of intestinal absorption of calcium. Transcellular absorption occurs in the duodenum and involves an active transport system, which depends on the presence of vitamin D. Paracellular absorption occurs mostly in the ileum and is passive. Lactose enhances transport at the paracellular level (*4*).

There have been many papers about the effect of dietary factors on calcium absorption. These factors include various types of dietary fiber (*5*, *6*), sugar alcohol (*7*), oligosaccharides (*8*, *9*), phosphorylated oligosaccharides (*10*), casein phosphopeptides (CPP) (*11*, *12*), and phosphopeptides derived from egg yolk phosvitin (*13*, *14*). The rate of absorption is also altered by several physiological factors. Age significantly affects the proportion of dietary calcium absorbed. Being female, experiencing menopause, some disease states, such as diabetes mellitus, and some medications lower the absorption of calcium (*15*). Calcium adequacy depends not only on the quantity of intake but also on its rate of absorption. Bioavailability refers to the portion of calcium in foods, which has been absorbed and is useable in the body. CPPs derived from tryptic digestion enhance the intestinal adsorption of calcium by facilitating paracellular absorption (*16*, *17*), and it has been authorized as

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a nutraceutical by the Ministry of Health and Welfare in Japan. Weakly binding a substantial amount of calcium and preventing calcium from being precipitated by certain substances can be effective for increasing calcium absorption in the body (*12*). Osteoporosis is a major public health problem today. It affects more than 25 million Americans and is responsible for 1.3 million new fractures of the spine, hip, and wrist in the U.S. each year (*18*, *19*). The magnitude of the problem is increasing dramatically as the elderly population continues to grow (*18*, *19*). Currently, estrogen replacement therapy is the most efficacious treatment to retard bone loss (*20*). Among several other factors, adequate calcium intake in association with vitamin D is crucial in the prevention and management of osteoporosis (*21*).

Chicken eggshell is a highly specialized mineralized structure that consists of approximately 95% calcium carbonate by weight, and of the remaining material, 3.5% is an organic matrix consisting mainly of glycoprotein and proteoglycans (*22*, *23*). Currently, eggshells are disposed of in many ways but are generally considered a waste product. Only recently has significant research been conducted into the possibility of the value-added utilization of eggshells as a food ingredient (*24*, *25*). Eggshell calcium has been proposed for pharmaceutical applications for calcium deficiency therapies in humans and in animals for bone mineralization and growth (*26*). Eggshell powder was shown to have antiraohitic effects in rats (*27*). *In vitro*, eggshell powder stimulated the growth of chick embryo cartilage cells (*28*). Use of the eggshell powder resulted in decreased pain and increased bone mineral density (*28*). In piglets, the apparent absorbability of calcium from eggshell powder was found to be at least as good as that from purified $CaCO_3$ (*29*). However, the factors resulting in increased bone mineral density have not been discovered. The eggshell consists of various organic matrix components. Numerous proteins that are present in various mineralized tissues and thought to be involved in the mineralization process possess calcium-binding properties (*30*). In chicken eggshell, it has been demonstrated that electrophoretic bands from insoluble or soluble eggshell extracts express calcium-binding properties (*31*, *32*).

In this study, we focused on eggshell matrix proteins as a potential factor to increase calcium transport. We isolated several soluble eggshell matrix proteins by reversed phase high-performance liquid chromatography (HPLC) and studied their effects on calcium transport in human intestinal epithelial cells using the Caco-2 cell line, which has been demonstrated as a powerful tool for *in vitro* transport studies of calcium and other minerals.

MATERIALS AND METHODS

Extraction of Soluble Eggshell Matrix Proteins. Eggs laid by 24−30 week old White Leghorn hens were supplied from the research unit at Arkell Poultry Research Station, University of Guelph, Ontario, Canada. Eggs were cracked, and eggshells were soaked in warm water overnight and then washed well to remove any traces of albumin. The collected shells were dried on the incubator at 37 °C after separating them from the eggshell membranes and ground to a fine powder using a mill. The extraction method of eggshell matrix proteins was adapted from Mann and Sieldler (*33*, *34*) with a slight modification. The eggshell powder was demineralized with 45% acetic acid (Fisher Scientific, Fair Lawn, NJ) with constant stirring at 4 °C overnight (100 g of powder per 1250 mL of acetic acid solution). The sample was placed in dialysis tubing (Spectrum Lab., Rancho Dominguez, CA, 1 kDa molecular mass cutoff) and dialyzed against Milli Q water for four nights at 4 °C. A precipitate that formed during dialysis was collected by centrifugation (8000*g* × 20 min), and the supernatant was lyophilized.

Protein Analysis. The concentration of protein in eggshell and extracted eggshell matrix proteins was analyzed using a nitrogen analyzer (Leco FP-528, Leco Instruments Ltd., Mississauga, ON).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed according to the method of Laemmli (*35*) in a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Samples were run on 15% homogeneous gels. Proteins were dissolved in sample buffer (1 M Tris-HCl, pH 6.8, containing 10% SDS) in the presence of 5% (w/v) β-mercaptoethanol, heated for 5 min at 95 °C in an Eppendorf Thermomixer 5436, and loaded onto the gel at 15 μg/well. Gels were run at a constant current (18 mA/slab gel), after which they were stained in 0.05% (w/v) Coomassie brilliant blue.

Cell Culture. Transwell polyester culture plates with a filter diameter of 12 mm and 0.4 μm pore size were obtained from Corning Costar (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco (Burlington, Ontario). Standard calcium solution (calcium chloride) was obtained from Sigma Chemical (St. Louis, MO). The flasks for growing cells were obtained from Corning Costar.

Caco-2 cell lines were used as a model of the intestinal epithelium, and this cell line was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM medium with 20% FCS, supplemented with 1% nonessential amino acids (Gibco) in the presence of penicillin and streptomycin (Gibco), and incubated at 37 °C in 5% CO_2. Passage numbers of 25−60 were used in all monolayers employed in calcium transport experiments. The medium was changed three times a week. Two generations of cells were grown on flasks for 15−20 days after seeding and then transferred to a transwell plate (2.0×10^5 cells/mL). The cells were cultivated for 14−21 days in DMEM with 20% FCS. The culture medium was 1.5 mL in the basolateral side and 0.5 mL in the apical side.

Transepithelial Electric Resistance (TEER) Measurement. The tight junction permeability of the Caco-2 monolayer was evaluated by measuring the TEER. The monolayers were checked routinely on alternate days by using Millicell-ERS system (Millipore, Bedford, MA). TEER values were found to be well-correlated with the change in paracellular permeability of the cell monolayer (*36*). TEER was derived from Ohm's law by measuring the potential difference change induced by an external current pulse. The TEER was measured before and after adding calcium sample.

Calcium Transport Studies. The transport experiments were performed with the transwell filters. The initial TEERs were 350−400 Ω cm^{-2}, and the integrity of the monolayer was determined. The monolayers were set in a 12 well plate and washed with Hank's balanced salt solution without calcium and magnesium (HBSS) twice after aspiration of the medium and incubated for 30 min at 37 °C with 5% CO_2 before the beginning of the calcium transport experiment. The wells were transferred to a new cluster plate containing HBSS buffer. At time 0, calcium solution (0−400 μg/well 0.5 mL) was added to the top compartment of the insert. Calcium transport rates were measured at different time intervals (10−240 min). The electrical resistance was monitored before and after incubation with calcium. After an optimum condition was fixed, the whole soluble eggshell matrix proteins (100 μg/well) or HPLC-isolated fractions (50 μg/well each) were added to the apical side with calcium solution. After incubation, both the apical and the basal samples were recovered for further analysis. Calcium uptake into cell monolayers was determined by recovering cells by solubilizing with 0.1 N NaOH and subjecting for calcium assay in the cells. Calcium contents were determined by atomic absorption spectroscopy with a Varian SpectrAA-300 (Melbourne, Australia).

Reversed Phase HPLC. Reversed phase chromatography was performed using Sephasil Protein C18 5 μm ST 4.6/250 or C4 5 μm ST 4.6/250 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in MilliQ water, and samples were eluted with a 0.085% (v/v) TFA in acetonitrile gradient (0−50%). Each fraction was dialyzed using 1 kDa cellulose membrane (Spectrum Lab.) against HBSS for 1 day and concentrated with a 1 kDa MWCO Centricon centrifugal filter (Millipore, Nepean, ON). All samples were subjected to calcium transport studies.

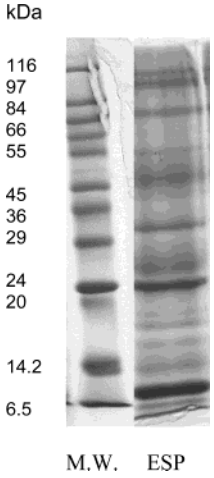

Figure 1. SDS–PAGE profile of soluble eggshell matrix proteins extracted by 45% acetic acid. MW, molecular marker; ESP, eggshell soluble matrix proteins.

N-Terminal Sequence Analysis. The N-terminal sequence analysis of purified eggshell matrix protein was carried out with a PE Applied Biosystems model 473A/494 Protein Sequencer (Foster City, CA).

Statistical Analysis. Data were analyzed by analysis of variance (SPSS version 8.0 for Windows; SPSS, Chicago, IL), and means were separated by Duncan's multiple ranges test. Significance was defined at $P < 0.05$.

RESULTS AND DISCUSSION

Extraction of Soluble Eggshell Matrix Proteins. In this study, eggshell matrix protein content was determined to be 0.89−1.11% (w/w), depending on individual eggs, by means of a nitrogen analyzer (data are not shown). This value was much lower than that reported previously (*22*, *23*). This might be because of overestimating of contaminant from albumen into eggshell membrane in early work. In 100 g of eggshell powder, an average of 54.4 mg of soluble proteins was obtained by the acetic acid extraction method as described previously (*37*). **Figure 1** shows the SDS electrophoretic pattern of the extracted soluble eggshell matrix proteins. It consisted of various bands between 10 and 100 kDa. In the past decade, biochemical characterization of chicken eggshell matrix proteins has been well-documented by various groups (*38*). Numerous proteins of the eggshell matrix have been identified. Ovocleidin-17 (17 kDa), a specific uterine protein, has been purified to homogeneity (*39*). Some of the eggshell matrix proteins were identical to those of egg white proteins (lysozyme, 14 kDa; ovalbumin, 45 kDa; and ovotransferrin, 76 kDa) (*39*). However, the electrophoretic profile of soluble eggshell matrix proteins seems to have a different pattern depending on the extraction methods. In this study, the 10 and 25 kDa proteins were predominant and the 17 kDa protein was a minor component.

Permeability of Caco-2 Cell Monolayer. Intestinal calcium absorption has been shown to include two processes, a saturable transcellular movement (active transport) and a nonsaturable paracellular pathway (passive transport). Paracellular transport is driven by external driving forces such as differences in concentration or electrical potential. It typically displays nonsaturable kinetics, cannot be inhibited by structural analogues, and does not require metabolic energy. Transcellular transport is still present in the absence of external driving forces. It should display saturation kinetics, show substrate specificity, function against a concentration gradient, and be dependent on metabolic energy. It can be driven directly either by ATPases

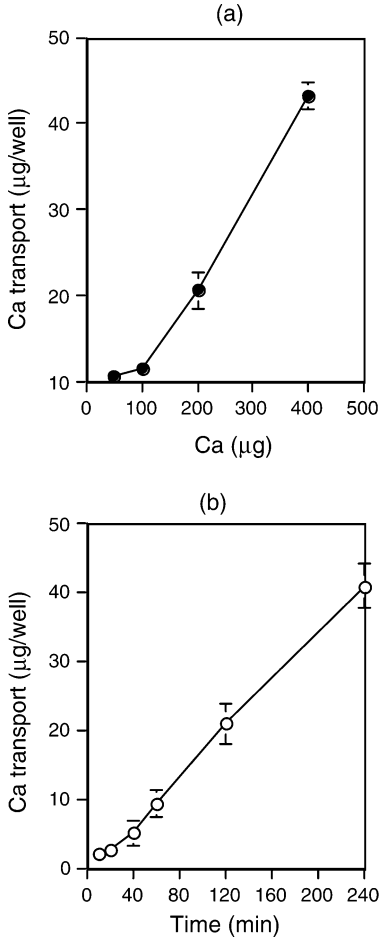

Figure 2. Calcium transport rates across Caco-2 cell monolayers as a function of dependence of calcium in 120 min of incubation (a) and time course with 200 μg Ca/well (b). Values reported are means ± SD in triplicates. Total calcium transport expressed as μg/well.

such as Na/K-ATPase or by flux coupling to a second substance, which undergoes primary paracellular transport (*40*). Transport processes in epithelia have traditionally been studied in a variety of organ and tissue preparations from different species. However, native epithelial tissues are complex and contain many different cell types. Monolayer cultures from established epithelial cell lines have the advantages of being structurally simple, homogeneous, and easy to manipulate (*41*). Caco-2, a transformed human colonic cell line, exhibits morphological as well as functional similarities to intestinal (absorptive) enterocytes (*42*). Caco-2 cells have been reported to be an excellent in vitro model of human enterocytes for the study of calcium transport (*43*). Monolayers of Caco-2 cells grown on a permeable support were used to assess the transport of calcium in the presence of soluble eggshell matrix proteins.

In our initial studies, the effect of calcium concentration and incubation time on the rate of transepithelial calcium transport by Caco-2 cells was determined. Calcium was added to the inner well bathing the apical cell surface, and the calcium on the basolateral side was evaluated at different calcium concentrations (**Figure 2a**) and different incubation time points (**Figure 2b**). The concentration dependence of transepithelial calcium transport in Caco-2 cells was found to be linear up to 400 μg/well added to the apical side. At 120 min, 10.1 (200 μg/well) and 10.8% (400 μg/well) calcium were transported across Caco-2 monolayers into the bottom compartment. We found the transport of calcium into the bottom compartment to

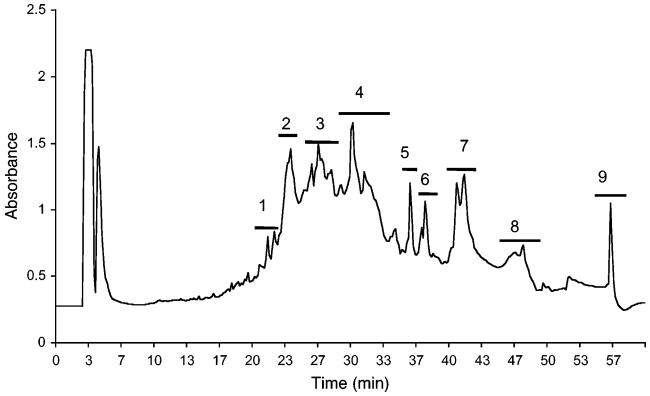

Figure 3. Reversed phase chromatographic profile of soluble eggshell matrix proteins. A 400 μg amount of eggshell proteins was injected to a Sephasil C18 ST 4.6/250 column, samples were eluted with a linear gradient (0–50%) of 0.1% TFA in water and 0.085% TFA/acetonitrile buffers at a flow rate of 1.0 mL/min, and a wavelength of 214 nm was used to monitor each peak.

Table 1. Effects of Isolated Soluble Eggshell Matrix Proteins on Calcium Transport Rates Across Caco-2 Cell Monolayers[a]

conditions	Ca transport (μg/well)	Ca in the cells (μg/well)	TEER (Ω/cm^2)
control ($n = 6$)	49.6 ± 3.8a	3.75 ± 0.3a	148 ± 4a
whole ESP ($n = 6$)	81.5 ± 4.2c	3.60 ± 0.38a	152 ± 8a
HPLC peak 1 ($n = 3$)	56.9 ± 3.1ab	3.30 ± 0.32a	156 ± 7a
peak 2 ($n = 3$)	55.9 ± 5.6ab	3.57 ± 0.27a	151 ± 5a
peak 3 ($n = 3$)	51.7 ± 3.2a	3.79 ± 0.49a	153 ± 6a
peak 4 ($n = 3$)	51.2 ± 3.3a	3.88 ± 0.82a	147 ± 4a
peak 5 ($n = 3$)	75.6 ± 2.4c	2.67 ± 0.38b	147 ± 5a
peak 6 ($n = 3$)	83.1 ± 6.3c	2.59 ± 0.49b	137 ± 9a
peak 7 ($n = 3$)	65.5 ± 4.1b	2.99 ± 0.62a	130 ± 13a
peak 8 ($n = 3$)	57.7 ± 2.9ab	3.11 ± 0.28a	139 ± 6a
peak 9 ($n = 3$)	53.2 ± 4.2a	3.02 ± 0.24a	135 ± 12a

[a] Means in the same column with different letters are significantly different ($P < 0.05$). The whole soluble eggshell matrix proteins (100 μg/well) or HPLC isolated fractions (50 μg/well) were added to the apical side with calcium solution (200 μg/well) and then incubated for 240 min.

be linear between 40 and 240 min at a calcium concentration of 200 μg/well. Thus, we fixed the subsequent experimental conditions at 200 μg calcium/well and an incubation time of 240 min to evaluate the effect of soluble eggshell matrix proteins on calcium transport.

Effect of Soluble Eggshell Matrix Proteins on Transepithelial Calcium Transport. As shown in **Figure 1**, the soluble eggshell matrix proteins consist of at least 14 bands with a wide range of molecular masses. The matrix proteins were separated into nine fractions by a Sephasil Protein reversed phase column (**Figure 3**). Each fraction was concentrated and dialyzed against HBSS for 1 day. **Table 1** summarizes the effect of eggshell matrix proteins (whole eggshell matrix proteins, 100 μg/well) and HPLC-isolated fractions (50 μg/well each) on calcium transport in Caco-2 cells. The whole soluble eggshell matrix proteins significantly increased calcium transport across Caco-2 monolayers. In the presence of whole eggshell matrix proteins, 64.3% more calcium was transported. Each HPLC peak (1−9) was also evaluated in the same manner. Peaks 3, 4, and 9 did not exhibit any significant increase in calcium transport, while peaks 1, 2, and 8 exhibited a slight increase. Interestingly, the calcium transport was significantly enhanced in the presence of peak 5 or 6 to the same level as whole mixtures of soluble eggshell matrix proteins. These results indicated that particular eggshell matrix proteins of peaks 5 and 6 have a biological function to increase calcium transport in Caco-2 cell monolayers. We also evaluated calcium uptake in cell monolayers grown on a permeable support in the presence of each fraction. Calcium accumulation in the cells after 240 min of incubation was low when compared to the total calcium found in the bottom compartment (i.e., 49.6 ± 3.8 calcium transport and 3.75 ± 0.3 in the cells in control, respectively). The calcium in the cells in the presence of peaks 5 and 6 was slightly lower than control. Other fractions did not affect the total calcium in the cells. There was no significant difference in TEER values under different conditions after 240 min of incubation. The TEER value in each well was not significantly changed at time 0 and 240 min after incubation (data are not shown).

Caco-2 cells are able to undergo spontaneous differentiation into enterocyte-like cells. Thereby, Caco-2 cells form confluent monolayers consisting of well-polarized cells with tight junctions and a typical apical brush (*42*). Caco-2 cell cultures are currently being utilized as *in vitro* models for absorption studies involving minerals (*44*), amino acids, protein, and peptides (*45*, *46*). The potential utility and advantages of Caco-2 cell monolayers for studying transepithelial intestinal calcium transport have already been demonstrated by various research groups (*40*, *47*, *48*). TEER is a highly sensitive parameter for membrane permeability. It is generally accepted that transepithelial electric conductance across Caco-2 monolayer is mainly determined by the ionic permeability of the intercellular junctions, which develop during postconfluent cell growth (*49*). Thus, the changing TEER largely reflects an effect of tight junction-mediated paracellular ion permeability. In this study, addition of soluble matrix proteins and their HPLC fractions did not change the TEER, while the total calcium transport into the basolateral side in the presence of peaks 5 and 6 increased. Our present results do not clearly indicate now by which way the isolated eggshell matrix protein (peaks 5 and 6) contributed to increase in calcium transport across Caco-2 monolayers. Uptake of calcium was not increased in the presence of peaks 5 and 6 matrix proteins.

Blais et al. (*50*) have demonstrated that the major route of calcium transport across Caco-2 cell monolayers is the paracellular way. The calcium transcellular transport represents a small percentage of total calcium transepithelial transport. It has been reported that vitamin D increases tight junction conductance and paracellular calcium transport in Caco-2 cells (*48*). CPP enhances the intestinal adsorption of calcium by facilitating paracellular absorption (*11*, *16*). At this point, the results obtained from this study do not permit a complete understanding of the increase of calcium transport across Caco-2 monolayers; however, it is obvious that the contribution of the transcellular pathway is small due to the insignificant increase of total calcium in the cells. We might need to introduce a more sensitive direct method for the study of tight junction permeability of calcium transport in the presence of eggshell matrix proteins. Various mechanisms have been reported as contributing to the absorption of calcium from the gut intestine. The paracellular pathway is well-substantiated; however, others are more controversial. It is not possible yet to sketch a clear picture of calcium transport at the cellular and molecular level (*40*). This should be addressed in future work.

Identification of Calcium Transport Enhancer in Eggshell Matrix Proteins. The HPLC peaks 5 and 6 were further subjected to purification by a Sephasil Protein C4 reversed phase column to identify the active protein enhancing calcium transport. Peak 5 was split into two major fractions (denoted a and b) and several minor components. Both peaks 5-a and -b

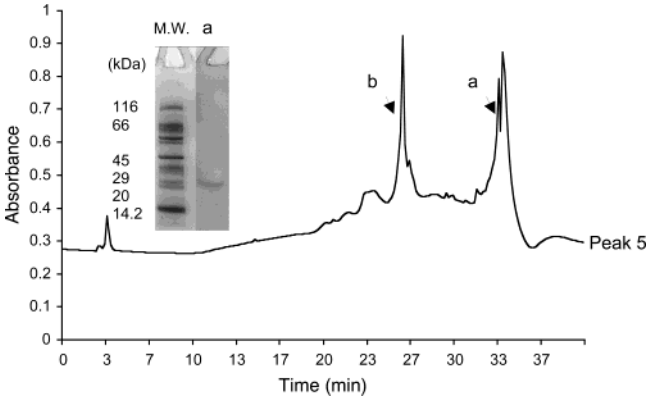

Figure 4. Purification of a soluble eggshell matrix protein by reversed phase chromatography. A 100 μg amount of peak 5 derived from **Figure 3** was injected to a Sephasil C4 ST 4.6/250 column, samples were eluted with a linear gradient (0–50%) of 0.1% TFA in water and 0.085% TFA/acetonitrile buffers at a flow rate of 1.0 mL/min, and a wavelength of 214 nm was used to monitor each peak. SDS–PAGE profile of a purified peak 5-a was shown.

shared a common peak with peak 6 (data are not shown). Therefore, we purified both peaks a and b until we got a single peak with homogeneity using the same column. The purified peaks a and b were evaluated for their ability to increase calcium transport across Caco-2 cells in the same manner performed in **Table 1**. Only peak a exhibited a remarkable increase in calcium transport in the presence of 25 μg/well of protein (91.2 $\pm$ 4.7 μg/well calcium transport), while peak b showed 54.7 $\pm$ 3.9 μg/well calcium transport, not significantly different from the control ($n = 3$). Therefore, we concluded that the major protein contributing calcium transport is peak a, with an estimated molecular mass of 21 kDa by SDS electrophoresis (**Figure 4**, inner picture). The protein is a novel protein that has never been identified in eggshell matrix proteins in the past based on an extensive search using the protein data bank (http://www.rcsb.org/pdb), and we mapped the N-terminal sequence to be Met-Ala-Val-Pro-Gln-Thr-Met-Val-Gln by means of an amino acid sequencer.

Some eggshell matrix proteins possess calcium-binding properties (*38*). Cortivo et al. (*51*) separated a low molecular mass acidic protein with calcium affinity, and this property was associated with a high content of aspartic and glutamic acid and a high content of sulfate groups. Similarly, ovotransferrin (78 kDa), ovalbumin (45 kDa), and others (36 kDa) express calcium affinity (*32*, *38*). It has also been demonstrated that CPP can weakly bind a substantial amount of calcium and prevent it from being precipitated by certain substances in the gut, improving calcium absorption (*16*, *17*). The physiological significance of calcium binding protein in the calcium transportation still remains unknown although theories and hypotheses are rampant. It might be of interest to investigate whether the identified 21 kDa protein possesses calcium-binding properties and its molecular role of increasing calcium transport across intestinal epithelial cells.

Eggshell calcium is proposed as an excellent calcium supplement in many health foods to increase bone mineral density in an elderly population with osteoporosis (*52*). However, its biochemical mechanism has not yet been evaluated. The present study clearly demonstrated that a 21 kDa protein in soluble eggshell matrix proteins plays an important role in increasing calcium transport across intestinal epithelial cells using *in vitro* Caco-2 cell monolayers. Many other minor components in eggshell such as calcitonin might also have a key role in the effect on bone mineral density (*52*, *53*). Further fundamental studies on the role of eggshell matrix components, in terms of their contribution to calcium transport, can bring new insight for better understanding of the nature of chicken eggshell and its value-added utilization for human health as nutraceuticals.

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