CHELATION of WALNUT PROTEIN PEPTIDE with CALCIUM and CALCIUM ABSORPTION PROMOTION in VIVO

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Abstract. In this study, walnut meal was used to prepare walnut protein. Papain was used to hydrolyze walnut protein to obtain active protein peptides. Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS) were used to study the chelating ability of walnut protein peptides and Ca²⁺ and complete structural characterization. A Caco-2 monolayer model and a rat calcium deficiency model were established to study the calcium absorption capacity and increase bone mineral density (BMD) of walnut protein peptide chelated calcium. The results showed that the appearance and internal structure of walnut peptides had changed after chelated with calcium. Infrared spectroscopy analysis indicated that walnut peptides may participate in the coordination of Ca²⁺ to form calcium chelating peptides through groups such as -COOH, -OH, and -NH₂. Caco-2 monolayer model experiments show that walnut peptides-Ca can promote the absorption and transport of calcium by regulating the TRPV6 signaling pathway. Calcium deficient rat model experiments show that the combination of walnut peptides and calcium can effectively increase BMD and improve bone formation, thereby preventing calcium deficiency. Walnut protein peptide chelated calcium could be a potential new product for calcium supplements.

1. Introduction
Calcium is an indispensable mineral element in animals and human bodies. It is involved in the body's signal transmission, muscle contraction, bone growth and other life processes[1]. Calcium deficiency is currently a global public health problem, especially in developing countries[2]. Calcium deficiency is initially asymptomatic and has no obvious impact on life, so it is easy to be ignored. If there is no timely intervention, long-term calcium deficiency will cause irreversible damage to the body, such as osteoporosis, hypertension, cardiovascular disease, kidney stones, and colon cancer[3-4]. In order to reduce the harm caused by insufficient calcium intake, in recent years, various calcium supplements have producted on the market, such as inorganic calcium, calcium gluconate, calcium amino acid. However, most of these calcium supplements are either low concentration in calcium or low in bioavailability and can cause side effects such as flatulence and bloating[5]. Therefore, when we
supplement the calcium, calcium bioavailability should be increased while calcium intake is increased, and healthy calcium supplementation should be emphasized.

The peptide calcium chelate has good solubility and can prevent calcium precipitation, thereby effectively increasing calcium absorption and promoting calcium utilization\cite{6}. At present, a variety of food protein peptides have been found and identified to bind calcium to promote calcium absorption, such as casein phosphopeptide\cite{7}, salted duck egg white peptide\cite{8}, tilapia peptide\cite{9}. Most of these peptides are derived from animals and milk products, which may not satisfy the needs of vegetarians and people allergic to animal dairy products. It has been reported that peptide chelated calcium is prepared from wheat, cucumber seeds and other plants, but the proportion of plant calcium supplement products available on the market is relatively small. Plant protein nutrients have a wide range of sources, low cost, and excellent performance. They have been favored as dietary nutritional supplements, and plant foods must be healthier\cite{10}. Therefore, there is an urgent need to increase the number of plant-derived calcium supplement products to provide consumers with more choices.

China's walnut cultivation and production rank first in the world\cite{11}. Except for some exports and domestic fresh food sales, walnuts are mainly processed into walnut oil, walnut powder, walnut milk and other products. During the production of walnut oil, a large amount of defatted walnut meal was produced. There are reports that the protein in walnut meal is as high as 53.89\%\cite{12}. At present, walnut meal is basically used as animal feed or even directly discarded, causing a lot of waste of resources. As we all know, walnut protein is a high-quality plant protein resource. Walnut protein contains 18 amino acids, including 8 essential amino acids, and the levels of arginine, glutamic acid, histidine, and tyrosine are relatively high, which are close to the standards set by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)\cite{13}. At present, there are many researches on the production of peptides based on the protein in walnut meal. However, the researches on walnut protein peptides mostly focus on process preparation\cite{14}, antioxidant\cite{15}, hypotensive\cite{16}, etc. It is no report about studies on chelation of walnut peptides and calcium to improve calcium bioavailability. The purpose of this study was to prepare walnut protein polypeptide and peptide calcium chelate by enzymatic hydrolysis. The expression of TRPV6 in Caco-2 monolayer under the action of walnut peptide calcium chelate was studied, and the effect of walnut peptide-Ca on the growth performance and bone metabolism of SD rats lacking calcium was investigated.

2. Materials and Method

2.1 Materials
Walnut meal powder was purchased from Yunnan Moemongzhuang Biotechnology Development Co., Ltd. Papain (\( \equiv 800,000 \) units/g) was purchased from Solibao Biological Co., Ltd., and ammonium sulfate was purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. The remaining reagents were of analytical grade.

2.2 Walnut Isolate Protein Extract
Some modifications were made to the methods of Sze-Tao\cite{16} and Sathe\cite{17}. After drying at 55 \(^\circ\)C to a constant weight, pass through a 150-mesh sieve of walnut meal, degrease with n-hexane (walnut meal/n-hexane ratio of 1: 10 w/v) for 1.5 h, and then filter under reduced pressure with filter paper until the filtrate is clear. The filter residue was ground and pulverized after being air-dried in a fume hood to obtain walnut meal defatted powder (DFWF), which was stored at -20 \(^\circ\)C until use.

Dissolved defatted walnut powder (DFWF) in distilled water (material-liquid ratio is 1: 20 w/v), adjust the pH to 9.0-9.5, after stirring for 2 h, centrifuge at 4 \(^\circ\)C, 1000 g, 30 min, the precipitate was dissolved again in distilled water adjusted to pH 11 (the ratio of precipitate to distilled water was 1:10 w/v), Stir for 1 h, centrifuge again at 1000 g for 30 min. Combine the two supernatants, adjust the solution to pH 4.5, let stand for 2 h at 4 \(^\circ\)C, then centrifuge at 1000 g for 30min at 4 \(^\circ\)C, collect the precipitate, wash the precipitate 3 times with distilled water with a pH at 4.5, the precipitate was reconstituted with distilled water, the pH was adjusted to 7.0, and dialysis was performed for 24 hours,
followed by lyophilization to obtain walnut protein isolate.

2.3 Preparation of walnut peptides
The obtained walnut protein was mixed at a ratio of 10 g of walnut protein to 150 mL of deionized water. Hydrolyzed with papain (55 °C, pH at 6) for 60 min, and the amount of enzyme added was 2% (referring to the quality of walnut protein). In order to inactivate the enzyme, after the hydrolysis was completed, the mixture was heated at 100 °C for 15 min. Subsequently, the enzyme solution was centrifuged at 4000 g for 20 min. The supernatant was collected and filtered through an ultrafiltration membrane (Millipore, Merck, USA) to obtain Walbut peptide with a molecular weight of less than 5 kDa. The peptide was freeze-dried and stored for later use.

2.4 Preparation of walnut protein peptide-Ca
The obtained walnut protein peptide was dissolved in deionized water (10 mg/mL). The mass ratio of peptide to calcium was 4:1, and the chelation temperature was 55 °C. The peptide and CaCl₂ were incubated for 60 min at a pH of 7.6. After chelation, anhydrous ethanol (six times the solution volume) was added to the reaction to isolate the peptide chelated calcium. Then it was centrifuged at 4000 rpm for 20 min, and the pellet was collected, lyophilized and labeled as walnut protein peptide-Ca.

2.5 Fourier transform infrared spectrum
The KBr tablet method was used to scan the infrared absorption between walnut peptide and walnut peptide calcium chelate between 4000 cm⁻¹ and 400 cm⁻¹ by using a Fourier transform infrared (FTIR) spectrophotometer (Perkin Elmer, MA, USA). Resolution is 4 cm⁻¹ and the number of scans is 16 times.

2.6 SEM and EDS
The sample was uniformly coated with a thin layer on an aluminum plate and gold-plated for 250 s. A scanning electron microscope (Nova NanoSEM 450, FEI, USA) was used for photo collection. The acceleration voltage was 20.0 kV. X-ray photoelectron spectroscopy (K-Massachusetts, USA) for elemental analysis.

2.7 Western Blot Analysis
Protein was extracted from Caco-2 cells by using RIPA lysis buffer (Solarbio, Beijing, China) with a protein loading of 50 μg per well. A 10% SDS-polyacrylamide gel was used to separate the proteins by electrophoresis under the conditions of 50 V voltage for 30 min and 120 V for 120 min, and then the proteins on the gel were transferred to a PVDF membrane under the conditions of 60 min wet rotation and 200 mA constant current (California Millipore, United States). It was then blocked with 5% skim milk for 1 h at room temperature, and then washed 3 times with 10 × PBST (10 min each). Primary antibodies to TRPV6 (Cambridge, USA) were incubated at 4 °C overnight. The PVDF membrane was washed 3 times (10 min each) with 1 × PBST, and incubated with a secondary antibody at a ratio of 1: 10,000 at room temperature for 60 min. After washing 3 times with 1 × PBST, protein bands were obtained.

2.8 Calcium Absorption and Femoral Features in Calcium-Deficient SD Rats

2.8.1 Animals and diets
All procedures for this animal experiment have been approved by the Animal Ethics Committee of Yunnan Agricultural University and meet all guidelines. Sprague Dawley (SD) rats weighing 60-80 g were obtained from Liaoning Changsheng Biotechnology Co., Ltd. [Certificate number: SCXX (Liao) 2015-0001]. The rats were housed at a temperature of 22 ± 2°C, a relative humidity of 60 ± 5%, 12 h of light and 12 h of darkness. All diets were purchased from Trophic Animal Feed High Tech Co., Ltd. (Nantong, China) and prepared according to the AIN-93 diet.
Seven days after the adaptation period, rats were randomly divided into normal control group, calcium deficiency group, CaCO₃ and walnut peptide calcium chelate low dose group (L-walnut peptide-Ca), walnut peptide calcium chelate high dose group (H-walnut peptide-Ca) (n = 10 in each group). The control group was fed a normal diet (calcium, 4500 mg/kg) for 8 consecutive weeks, and the remaining groups were fed a calcium-deficient diet (calcium, 100 mg/kg) for 8 consecutive weeks. The daily intake of L-walnut peptide-Ca group was 200 mg/kg and L-walnut peptide-Ca group was 400 mg/kg body weight, respectively. Both CaCO₃ and L-walnut peptide-Ca group were administered by gavage (The dose is equivalent to the human recommended calcium intake of 800 mg/kg b.w).

2.8.2 Calcium absorption experiment
The experiment was carried out for last three days of week 4 for conducting calcium metabolism experiments. In the last 3 days of the experiment, rat feces were collected and weighed daily, and calcium excreted in the feces was measured. The absorption rate calculation was done as follows:

- Intake calcium (mg/d) = calcium content in feed (mg/g) × feed consumption (g/d).
- Faecal calcium (mg/d) = faecal calcium content (mg/g) × faeces excretion (g/d).
- Calcium apparent absorption rate = (intake of calcium - faecal calcium) / intake of calcium × 100%.

2.8.3 Sampling and testing
After a 8-week feeding period, all rats were fasted overnight and anesthetized with ether. The femoral bone mineral density (BMD) was quickly measured by using a dual-energy x-ray bone densitometer. The blood collected from the abdominal aorta was centrifuged to separate the serum. The serum Ca, P, alkaline phosphatase (ALP) levels were measured by using a commercial kit (Nanjing Institute of Bioengineering, China). After the blood was taken, the rats were sacrificed by cervical dislocation. The heart, liver, spleen, lungs, and kidneys were excised and then weighed to calculate the visceral index. The left and right femurs were anatomized, and the adherent tissue was eliminated. After removing the surrounding muscles completely, the resulting femur weight was weighed. All left femurs were dried, ground, and dissolved in 5% nitric acid by using an inductively coupled plasma optical emission spectrometer (10-ES, Varian, USA) to detected bone calcium content. The organ coefficient was then calculated as follows:

Coefficient of each organ = weight of each organ / body weight
Organ coefficient of wet femur = wet weight of the femur / body weight.

3. Results and discussion

3.1 FTIR Spectral Analysis
FTIR can effectively distinguish the difference of spectral between two substances. It is often used to evaluate the formation and composition of organic functional groups, mainly including O-H, C-O and N-H. As shown in Figure 1A, after Walnut peptide is chelated with Ca²⁺, some changes are found in the FTIR spectrum. The absorption peak at 3306.64cm⁻¹ is red-shifted to 3306.12cm⁻¹, which may be caused by the coordination between -NH₂ and Ca²⁺. N-H extends while hydrogen bond is replaced by Ca²⁺ bond. The wave number between 1475 cm⁻¹ and 1000 cm⁻¹ is mainly the X-H plane bending vibration and X-Y telescopic vibration zone. After chelation, a blue shift of 1452.51 cm⁻¹ occurred to 1441.44 cm⁻¹, and a red shift of 1389.82 cm⁻¹ reached 1402.69 cm⁻¹, a new absorption peak appeared at 1396.39 cm⁻¹. In addition, the wavenumbers at 1314.78 cm⁻¹, 1231.98 cm⁻¹ and 1185.88 cm⁻¹. The absorption peaks at 1177.55 cm⁻¹ and 1154.91 cm⁻¹ disappeared. The absorption peaks at 1130.61 cm⁻¹ and 1078.37 cm⁻¹ blue shifted to 1079.59 cm⁻¹ and 1049.88 cm⁻¹, respectively. This type of chelation may be due to carbonyl oxygen and Ca²⁺ being chelated by unbound free electrons to form COOCa. In addition, the red-shift, blue-shift, and disappearance of absorption peaks appeared in multiple absorption peaks with wave numbers between 1000 cm⁻¹ and 500 cm⁻¹, which may be mainly caused by the vibration of C-H and N-H bonds and being replaced by N-Ca. As
described above, the oxygen atom on the carboxyl group, the nitrogen atom on the amino group, and the hydrogen on the peptide bond are the interaction sites, which play a major role in the chelation reaction between Walnut peptide and Ca$^{2+}$. Each substance has its own FTIR spectrum, but the peak positions of the functional groups are basically the same. Similar results have been obtained in studies of calcium chelates such as cucumber peptide and whey peptide$^{18,19}$.

3.2. SEM and EDS analysis

Fig.1B shows the microstructures of Walnut peptide Fig.1 B1, B3 and Walnut peptide-Ca Fig.1 B2, B4. It can be seen from the figure that the surface of Walnut peptide and Walnut peptide-Ca are both porous structures, but the surface of Walnut peptide has larger pores. Walnut peptide-Ca has smaller pores on the surface and a looser structure, and a loose particle structure appears on the surface. This microstructure change may be caused by the interaction between Walnut Peptide and calcium, which destroys the original dense structure of Walnut Peptide. As stated by Liu et al., the carboxyl and amino groups in the polypeptide are combined with Ca$^{2+}$ to form a "bridge", which also changes its characteristics$^{20}$. Further research was performed by using EDS to study in detail the internal distribution of calcium in Walnut peptide and Walnut peptide-Ca. The analysis results are shown in Figures 1C, D. Both Walnut pepetid and Walnut pepetid-Ca contain calcium, carbon,
Fig. 1. A is Fourier spectrum spectra of walnut peptide and walnut peptide-Ca; B1 and B3 are SEM of walnut peptide (5000×, 2,5000×), B2 and B4 are SEM of walnut peptide-Ca (5000×, 2,5000×); Figure C is EDS analysis of walnut peptide and walnut peptide-Ca, C1 is walnut peptide, C2 is walnut peptide-Ca.

nitrogen, sodium, and other elements. This result shows that the calcium signal intensity after chelation is significantly higher than that before chelation. The data are shown in Table 1. It further shows that Walnut peptide can chelate with Ca\(^{2+}\) and has good chelating ability.

| Sample          | The content of calcium (%) |
|-----------------|-----------------------------|
| Nut Peptide     | 2.16 ± 0.91                 |
| Nut Peptide-Ca  | 17.01 ± 1.99                |
3.3 Effect of Walnut peptide-Ca on calcium absorption in SD rats of calcium deficiency

Table 2 shows the calcium intake in the last 3 days of the fourth week, the apparent absorption rate of calcium and the calcium retention rate in different groups. The results showed that the absorption rate and retention rate of calcium in the calcium deficiency group were significantly higher than those in the other experimental groups. It may be due to the lack of long-term calcium intake in the calcium-deficient group, and the body is in a “calcium-deficient” state, so the absorption and retention of calcium is significantly higher than in the other experimental groups. This result is also similar to that of Welch AA et al. \cite{21}. It can be seen from Table 2, the calcium absorption rate and storage rate of the L-Walnut Peptide-Ca group were significantly higher than those of the control group and the CaCO$_3$ group ($P < 0.05$), the H-Walnut Peptide-Ca group were significantly higher than that of the CaCO$_3$ group, but there was no significant difference from the control group ($P > 0.05$).

Inorganic calcium easily interacts with food ingredients and forms a precipitate in the body, so it is difficult to absorb and easy to excrete\cite{22}. Calcium absorption in the digestive tract includes active transport and passive diffusion. When the calcium intake is low, active transport accounts for the major part of calcium absorption and the calcium absorption rate is higher. When the calcium intake is high, passive diffusion accounts for the major part of calcium absorption. Passive diffusion depends on the osmotic pressure difference and the solute concentration difference on both sides of the absorption membrane, and it follows the concentration gradient without energy consumption. The calcium absorption rate is less than 10%. Active transport mainly occurs in the duodenum, while passive diffusion mainly occurs in the large intestine. Therefore, when the calcium intake is too low, active calcium transport accounts for the vast majority, and the absorption rate of calcium rises rapidly, which increases the absorption rate of calcium. When the calcium intake is too high, the active absorption of calcium has tended to be saturated, and passive diffusion accounts for the main part, and a calcium absorption platform effect will occur, resulting in a decrease in calcium absorption rate.

![Graph](image)

Fig. 2. A shows the changes in body weight of calcium-deficient rats in different groups, and B shows the ratio of organ coefficients in different groups.

3.4 Effect of Walnut peptide-Ca on the signs and serum Ca, P, ALP of SD rats with calcium deficiency

During feeding, the rats did not show any abnormal conditions, such as death and diarrhea. Figures 2A and B show the data of body weight change and visceral (heart, spleen, lung, kidney, liver) index of rats in different groups after 8 weeks of feeding. It can be seen from the figure that the body weight of the rats did not change significantly in the 4 weeks before feeding. After 4 weeks, the rats in the calcium-deficient group gradually showed a downward trend, but there was no significant difference between the groups ($P > 0.05$). Until the eighth week, the body weight of the rats in the
calcium-deficient group was significantly lower than that in the control group ($P < 0.05$), and the weight of the other experimental groups increased, but there was no significant difference$^{[23]}$. Early calcium-deficient organisms do not change significantly and are therefore easily overlooked. Fig. 2B shows that there is no significant difference between the organ coefficients of rats in different groups. This shows that Walnut Peptide not affect normal growth and health of the body, it is safe.

Table 3 shows some serum biochemical indicators related to calcium metabolism. ALP is a marker of bone formation and metabolism and plays a vital role in bone calcification. As shown in Table 3, ALP activity increased significantly when calcium intake was insufficient.

Table 2. Absorption rates of normal and calcium-deficient mice after different Ca treatments.

| Calcium absorption (%) | Normal | Calcium deficiency | L-walnut Peptide Ca | H-walnut Peptide Ca |
|------------------------|--------|--------------------|--------------------|--------------------|
| Calcium intake (mg/day) | 71.76 ± 6.93 | 4.63 ± 0.94 | 36.98 ± 4.92 | 36.97 ± 4.92 |
| Ca absorption (%) | 57.58 ± 3.37a | 89.87 ± 2.95b | 45.98 ± 3.01c | 74.81 ± 4.82d |
| Ca retention (%) | 54.21 ± 2.44a | 83.47 ± 3.01b | 43.99 ± 3.12c | 72.05 ± 5.35d |

Data are expressed as mean±standard deviation (n = 10). Any two means in the same row followed by the same letter are not significantly different ($P > 0.05$).

Table 3. Femur properties and biochemical properties (Ca, P, ALP) of serum in different groups.

| Measurement                  | Normal          | Calcium deficiency | CaCO$_3$ | L-walnut Peptide Ca | H-walnut Peptide Ca |
|------------------------------|-----------------|--------------------|----------|--------------------|--------------------|
| Serum biochemistry           |                 |                    |          |                    |                    |
| Ca (mmol/L)                  | 3.02 ± 0.18a    | 2.85 ± 0.23a       | 3.05 ± 0.14a | 2.96 ± 0.17a       | 2.95 ± 0.13a       |
| P (mmol/L)                   | 4.01 ± 0.44a    | 4.55 ± 0.51a       | 4.80 ± 0.64a | 4.91 ± 0.18a       | 4.89 ± 0.76a       |
| ALP (U/L)                    | 82.60 ± 7.79a   | 113.80 ± 8.47b     | 93.22 ± 9.57a | 93.85 ± 11.56a     | 89.27 ± 13.42a     |
| Femur indices                |                 |                    |          |                    |                    |
| Weight (mg/g)                | 3.83 ± 0.394a   | 3.26 ± 0.38b       | 3.31 ± 0.62b | 3.54 ± 0.26ab      | 3.64 ± 0.25ab      |
| Ca content (mg/g)            | 152.37 ± 5.43a  | 130.17 ± 6.26c     | 137.98 ± 6.13bc | 142.18 ± 3.38b     | 147.73 ± 5.13a     |
| Femur BMD (g/cm$^2$)         | 0.22 ± 0.02a    | 0.13 ± 0.01d       | 0.16 ± 0.01c | 0.17 ± 0.02bc      | 0.18 ± 0.02b       |

Data are expressed as mean±standard deviation (n = 10). Any two means in the same row followed by the same letter are not significantly different ($P > 0.05$).

Although there were no significant differences in serum ALP levels between the control group, the CaCO$_3$ group, and the Walnut peptide-Ca group, compared with the calcium-deficient group, the serum ALP levels were significantly reduced ($P < 0.05$). This result is also similar to the results of Peng et al.$^{[23]}$ study of cod bone peptide calcium chelation and calcium absorption promotion. The results of serum Ca test showed that although the calcium content of the calcium deficiency group had a decreasing trend, the calcium content were not statistically significant among the groups ($P > 0.05$). And there was no significant change in serum phosphorus content($P > 0.05$). Insufficient long-term calcium intake, in order to maintain a relatively stable blood calcium concentration, the body uses the calcium in the "bone bank" and releases it into the blood to regulate the blood calcium balance. This may cause a decrease in bone calcium.
3.5 Effect of Walnut peptid-Ca on femoral characteristics in SD rats with calcium deficiency
As shown in Table 3, at the end of the feeding period, the femoral wet weight of the calcium-deficient group and the CaCO$_3$ group was significantly lower than that of the control group ($p < 0.05$). The Walnut peptide-Ca groups showed an increasing trend compared with the calcium deficiency group. Although there was no significant difference, it was close to the normal group. After feeding Walnut Peptide-Ca at different concentrations, the bone calcium content was significantly higher than that of the calcium deficiency group ($P < 0.05$), and higher than that of the CaCO$_3$ group, tending to the normal group. This result also corresponds to the result of serum calcium content. In addition, the BMD of rats with chronic calcium deficiency was significantly reduced, and then different doses of walnut peptide-Ca could effectively prevent the decrease of bone density, and the effect of walnut peptide-Ca group was significantly better than CaCO$_3$ group ($P < 0.05$), which was close to the normal group. This result is similar to some previously reported calcium supplements that can promote bone health$^{[26]}$. This result showed that Walnut Peptide-Ca is beneficial to the bone growth of calcium-deficient rats. It can promote the deposition of calcium in the bones of rats, and has the effect of preventing bone loss caused by calcium deficiency.

3.6 Effect of pepetid-Ca on the expression of TRV6 protein in Caco-2 cells in vitro
The small intestine is the main site for Ca$^{2+}$ absorption, and 90% of calcium is absorbed through the small intestine$^{[27]}$. The calcium transported by the peptide through the intestinal epithelium mainly includes the transcellular pathway and the paracellular pathway. TRPV6 is a pathway in the transcellular pathway, a protein channel located in the epithelial cell membrane of the intestinal wall, can promote the absorption of calcium ions in the diet, and is a highly selective Ca$^{2+}$ channel in the members of the TRPV ion channel family. It plays an important role in the absorption of Ca$^{2+}$ in the intestine$^{[28]}$. In order to further explore the mechanism of Walnut peptide regulating calcium absorption, the effect of TRPV6, a key calcium transporter protein, was analyzed after Walnut peptide acted on Caco-2 cells. The results are shown in Fig.3. After Walnut peptide and Walnut peptide-Ca treatment, the expression of TRPV6 protein increased significantly, and the expression was higher than that of CaCl$_2$ group. This finding suggests that Walnut peptide may act on the TRPV6 calcium channel and promote the effect of improving absorption.

![Fig.3](image)

Fig.3 A is a Western blot of TRPV6, and B is a map of relative expression.

4. Conclusion
Polypeptides prepared from walnut protein that extracted from walnut meal can efficiently bind
calcium ions to form polypeptide chelated calcium. Fourier analysis, scanning electron microscopy, and EDS results showed that the coordination groups of the walnut protein peptide and calcium chelate might be -COOH, -OH, and -NH₂. In vitro Caco-2 cell model and in vivo SD rat animal experiments show that walnut peptides promote the intercellular transport of Ca²⁺ by regulating the TRPV6 signaling pathway. At the same time, compared with calcium carbonate, it can more effectively increase the bone calcium content and bone density of calcium-deficient rats. Walnut protein peptide chelated calcium can be a potential new product for calcium supplements, and it can also provide new research ideas for the utilization of walnut protein and new product development.

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Reference
[1] G.D. Miller, J.K. Jarvis, L.D. McBean, The importance of meeting calcium needs with foods, J Am Coll Nutr, 20 (2001), 168s-185s.
[2] T. Fujita, Calcium paradox: Consequences of calcium deficiency manifested by a wide variety of diseases, Journal of Bone & Mineral Metabolism, 18(2000), 234-236.
[3] G.H. Hofmeyr, S. Manyame, M. Medley, M.J. Williams, Calcium supplementation commencing before or early in pregnancy, or food fortification with calcium, for preventing hypertensive disorders of pregnancy, Cochrane Database of Systematic Reviews, 16 (2017).
[4] M. Cozzolino, D. Brancaccio, M. Gallieni, A. Galassi, E. Slatopolsky, A. Dusso, Pathogenesis of parathyroid hyperplasia in renal failure, J Nephrol, 18 (2005), 5-8.
[5] L.D. Guo, A. Padraign, Harnedy, B.L. Lia, H. Hou, Z.H. Zhang, Food protein-derived chelating peptides: Biofunctional ingredients for dietary mineral bioavailability enhancement. Trends in Food Science & Technology, 37(2014), 92-105.
[6] J. Beeuer, M. Casutt, E. Stiksa, P. Vassilakos, H. Voegeli, Treatment of condylomata of the cervix uteri with local administration of lysozyme, Revue suisse de medecine Praxis, 74(1985), 1337-9.
[7] H. Meisel, H. Frister, Chemical characterization of a caseinophosphopeptide isolated from in vivo digests of a casein diet, Biological Chemistry Hoppe-Seyler, 369(1988), 1275-1280.
[8] T. Hou, W. W, W. Shi, Z.L. Ma, H. He, Desalted duck egg white peptides promote calcium uptake by counteracting the adverse effects of phytic acid, Food Chemistry, 219(2017), 428-435.
[9] J. Chen, X. J. Qiu, G.X. Hao, M. Zhang, W.Y, Weng, Preparation and bioavailability of calcium-chelating peptide complex from tilapia skin hydrolysates, J Agric Food Chem, 97(2017), 4900-4903.
[10] S.Rousseau, C. Kyomugasho, M. Celus, MEG. Hendrickx, T. Grauwet, Barriers impairing mineral bioaccessibility and bioavailability in plant-based foods and the perspectives for food processing, Crit Rev Food Sci Nutr, 60(2020), 826-843.
[11] J. Tian, Y. Wu, Y. Wang, F. Han, Development and prospects of the walnut industry in china, Acta Horticulturae, (2010), 31-8-38.
[12] W.G. Kou, H.Q. Gao, Development and Utilization of Walnut Products, China Oils, 6(2000), 111-112.
[13] X. Mao, Y. Hua, G. Chen, Amino Acid Composition, Molecular Weight Distribution and Gel Electrophoresis of Walnut (Juglans regia L.) Proteins and Protein Fractionations, International Journal of Molecular Sciences, 15(2014), 2003-2014.
[14] L. Cuiting, M. Fangli, W. Tingting, Preparation of defatted walnut meal hydrolysate-loaded enteric-coated pellets with enhanced oral absorption efficiency, Journal of Drug Delivery Science and Technology, 46(2018), 207-214.
[15] W. Wu, S. Zhao, C. Chen, F. Ge, D. Liu, X. He, Optimization of production conditions for antioxidant peptides from walnut protein meal using solid-state fermentation, *Food Science and Biotechnology*, 23(2014), 1941-1949.

[16] C. Wang, M. Tu, D. Wu, H. Chen, C. Chen, Z. Wang, L. Jiang, Identification of an ACE-Inhibitory Peptide from Walnut Protein and Its Evaluation of the Inhibitory Mechanism, *Int J Mol Sci.*, 19(2018), 1156.

[17] K.W.C. Sze-Tao, S.K. Sathe, Walnuts (Juglans regia L): Proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility, *J Agric Food Chem.*, 80(2000), 1393-1401.

[18] N. Sun, Z. Jin, D. Li, H. Yin, S. Lin, An Exploration of the Calcium-Binding Mode of Egg White Peptide, Asp-His-Thr-Lys-Glu, and In Vitro Calcium Absorption Studies of Peptide-Calcium Complex, *J Agric Food Chem.*, 65(2017), 9782-9789.

[19] S.Y. Wang, J.P. Lin, X.X. Cai, M.R. Tang, S.Y. Wang, Preparation and Evaluation of the Chelating Nanocomposite Fabricated with Marine Algae Schizochytrium sp Protein Hydrolysate and Calcium, *J Agric Food Chem.*, (2015).

[20] T. Hou, W. W, W. Shi, Z.L. Ma, H. He, Desalted duck egg white peptides promote calcium uptake by counteracting the adverse effects of phytic acid. *Food Chemistry*, 219(2017), 428-435.

[21] A.A. Welch, A.C. Hardcastle, The effects of flavonoids on bone, *Curr Osteoporos Rep*, 12(2014), 205-210.

[22] R. Liang, Y. Jiang, W.H. Yokoyama, C. Yang, G. Cao, F. Zhong, Preparation of stable Pickering emulsions with short, medium and long chain fats and starch nanocrystals and their in vitro digestion properties, *RSC Advances*, 6(2016), 99496-99504.

[23] D. Chen, X.M. Mu, H. Huang, R.Y. Nie, Y. Z. Liu, Y.M. Zeng, Isolation of a calcium-binding peptide from tilapia scale protein hydrolysate and its calcium bioavailability in rats, *Journal of Functional Foods*, 6 (2014), 575-584.

[24] X. Hu, P. Zhang, Z. Xu, H. Chen, X. Xie, GPNMB enhances bone regeneration by promoting angiogenesis and osteogenesis: Potential role for tissue engineering bone. *Journal of Cellular Biochemistry*, 114, (2013), 2729-2737.

[25] Z. Peng, H. Hou, K. Zhang, B. Li, Effect of Calcium-binding Peptide from Pacific Cod(Gadus macrocephalus) Bone on Calcium Bioavailability in Rats, *Food Chemistry*, 221(2017), 373-378.

[26] B. Waeber, H. Brunner, Calcium deficiency in the elderly: a factor contributing to the development of hypertension, *European Journal of Endocrinology*, 130(1994), 433-433.

[27] W.K. Jung, B.J. Lee, S.K. Kim, Fish-bone peptide increases calcium solubility and bioavailability in ovariectomised rats, *British Journal of Nutrition*, 95(2006), 124.

[28] T. Nijenhuis, J.G.J. Hoenderop, J.M.B. René, TRPV5 and TRPV6 in Ca²⁺(re)absorption: regulating Ca²⁺entry at the gate, *Pfluegers Archiv European Journal of Physiology*, 451(2005), 181-192.