Purification of a copper binding peptide from the mushroom *Grifola frondosa* and its effect on copper absorption

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The present studies were undertaken to investigate the effect of a copper binding peptide on copper bioavailability. This peptide was extracted from the fruit bodies of the mushroom, Grifola frondosa, *and purified to a single peptide using gel filtration and ion exchange chromatography. The purified copper binding peptide was an acidic peptide of molecular weight 2,240 in which four amino acids (aspartic acid, glutamic acid, serine, and glycine) occupied about 84% of total residues, and possessed specific properties to bind copper or to maintain copper in the soluble state at physiological pH. Molar ratio of the peptide and copper binding was 1.01. This peptide, when added to the mucosal solution in the in vitro experiment using everted intestinal sac of the rat, could significantly increase the amount of copper transported across the intestinal cells, compared with the addition of casein or its digestive peptides. These data suggest that this peptide enhances copper absorption in the small intestinal tract by increasing the amount of soluble copper.*

Keywords: copper; peptide; absorption; mushroom

Introduction

It is generally recognized that proteins or peptides have various binding activities for cations, and the degree of affinity for cations is greatly influenced by their amino acid composition and sequence. There have been many reports showing dependence of mineral absorption on dietary proteins[1–4] or free amino acids.[5–8] However, little evidence is available on the fact that the direct interaction between dietary proteins and minerals can be better understood by clarifying the definite effect of a peptide found in food or formed during the digestion of its protein.[9] Especially in the relationship between copper absorption and peptides, there are many ambiguous points, in spite of their high chelating activity of copper.[10,11] Therefore, it is nutritionally significant to investigate the physiological function of peptides on the copper bioavailability, their structural characteristics, and binding properties.

Heavy metals accumulating in some mushrooms are well known in the field of the toxicology or environmental sciences.[12] Edible mushrooms, which are rich in minerals and supply many nutrients, have not been well investigated with regard to their nutritional or physiological functions. Therefore, we previously studied this edible mushroom with particular interest in examining the effect of copper binding peptides found in its fruit bodies on copper absorption, and elucidating the mechanism of the interaction between copper and its binding peptide. Accordingly, in the present studies we attempted to purify the copper binding peptide in the fruit bodies of the mushroom, *Grifola frondosa,* which contained the highest amount of copper compared with other Japanese mushrooms, such as *Pleurotus ostreatus* and *Lentinus edodes,* and to study its function for copper bioavailability.

Materials and methods

The fruit bodies of the artificially cultivated mushroom *Grifola frondosa* (Japanese name: Maitake) were purchased

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from local grocery stores. Sephadex G-50 was purchased from Pharmacia (Piscataway, NJ USA). DEAE-Cellulose (DE-32) was from Whatman (Clifton, NJ USA). HPLC column, Asahipak GS-320, was from Asahi Chemical Industry Co., Japan. BCA (bicinchoninic acid) Protein Assay kit was obtained from Pierce Chemical Co. (Rockford, IL USA). Dialysis tubing was purchased from Spectrum Medical Industries Inc., USA. All other chemicals used were of the highest grade of purity commercially available.

All the purification procedures of the copper binding peptide from *G. frondosa* were carried out at 4° C. To avoid air oxidation during purification, all buffers used were previously saturated with nitrogen gas. The washed fresh fruit bodies of *G. frondosa* (300 g wet wt.) were sliced and homogenized with 300 mL of 20 mmol/L Tris-HCl buffer (pH 8.0) containing 2.0 mmol/L dithiothreitol(DTT), and then centrifuged at 17,000*g* for 30 minutes. The supernatant obtained was applied to a Sephadex G-50 column (6 × 105 cm) equilibrated with 20 mmol/L Tris-HCl buffer (pH 8.0) and eluted with the same buffer. The eluted low molecular weight (MW) fractions, rich in copper, were combined and immediately applied to a DEAE-Cellulose (DE-32) column (2.4 × 7.0 cm) equilibrated with 20 mmol/L Tris-HCl buffer (pH 8.0). After washing the column with the same buffer, it was eluted with a linear gradient of NaCl in the buffer from 0–0.3 M. The eluted copper binding fractions were immediately lyophilized after dialysing against 10 mmol/L ammonium bicarbonate (pH 8.0) using dialysis tubing (Spectra/Por6, MW cutoff:1000) and again dissolved in the same solution. Further purification of the copper binding peptide was performed by gel filtration on a Sephadex G-50 column (1.5 × 100 cm) equilibrated with 10 mmol/L ammonium bicarbonate. The eluted fractions containing the copper binding peptide were collected and lyophilized immediately.

The protein concentration was determined by using the BCA protein assay kit. The copper analysis was performed using atomic absorption spectrophotometer AA-640-13 (Shimadzu, Japan).

The homogeneity of the purified copper binding peptide was established by a multimode HPLC using an Asahipak GS-320 column (0.75 × 50 cm), at the flow rate of 0.5 mL/min at 30° C. The buffer system was 50 mmol/L ammonium acetate (pH 8.0) and the absorbance was recorded at 220 nm.

The copper binding peptide was rendered metal free by dialysis against 0.01 M HCl for 6 hours at room temperature, followed by gel filtration on Sephadex G-25 (1.5 × 40 cm) in 0.01 M HCl.

To investigate the amino acid composition of the copper binding peptide, the lyophilized apoprotein was hydrolyzed in 6 M HCl for 22 hours at 110° C in a vacuum-sealed glass tube, and then applied to an amino acid analyzer L-8500 (Hitachi, Japan). Just before or after hydrolysis of the peptide, the determination of amino groups with TNBS (2,4,6-trinitrobenzensulfonic acid) was performed using the method of Fields[13] to calculate the chain length of the peptide. The apparent molecular weight of the copper binding peptide was estimated by gel filtration following the method of Andrews[14] on a Sephadex G-50 column (1.0 × 50 cm) equilibrated with 50 mmol/L Tris-HCl buffer (pH 8.0) containing 0.1 M KCl.

The effect of the copper binding peptide on copper solubility in phosphate buffers of variable phosphate concentration were determined as follows. To 0.45 mL of Na-phosphate buffer (pH 7.0), ranging in final concentration from 0–40 mmol/L containing either 0.5 mg of the copper binding peptide or none, was added 0.05 mL of 25 mmol/L $CuSO_4$ and the solution was rapidly mixed. After standing for 2 hours, the resulting insoluble copper was removed by centrifugation at 3,000*g* for 10 minutes, and the copper content in the supernatant was measured with atomic absorption analysis.

To examine the effect of peptide concentration on copper solubility in a buffer with constant phosphate concentration, 0.05 mL of 25 mmol/L $CuSO_4$ and 0.05 mL of 50 mmol/L Na-phosphate buffer (pH 7.0) were added to 0.4 mL of the copper binding peptide solution ranging in final concentration from 0–1.2 mg/mL and mixed well. After standing for 2 hours, the soluble copper content was determined as above.

The effect of pH on the stability of the copper-peptide complex was also determined using an equilibrium dialysing method.[15] The copper binding peptide was dialyzed against 20 mmol/L HCl-KCl buffer (pH 2.0) for 12 hours at room temperature to remove copper ions from the peptide using dialysis tubing (Spectra/Por6, MW cutoff: 1000), and then equilibrium dialysis was performed in the above cellulose tubes using a continual equilibrium dialysing cell (Sanplatec Co., Osaka, Japan). Each dialysis was performed in cells containing 0.5 mL of apoproteins (16.7 μg/mL) or blank solutions, against 20 mmol/L buffers of several types for 20 hours at room temperature, and after the addition of 5 μg of copper ions (as $CuSO_4$) in cells, further dialysis was run against the same buffer for 20 hours. The copper content of the inner solution was determined as described above.

The effect of the copper binding peptide on copper transport by the rat was determined by using an in vitro everted intestinal sacs technique. Wistar male rats were fed a commercial diet. Ileal segments of rats fasted for 24 hours were used. The everted intestinal sac was prepared by the method of Barr and Riegelman.[16] The mucosal solution contained 122 mmol/L NaCl, 5 mmol/L KCl, 26 mmol/L $NaHCO_3$, 1 mmol/L KH_2PO_4, 1.2 μmol/L $CuSO_4$, and 16.7 μg/mL of the copper binding peptide or other peptides. The pH was adjusted to 7.4. The volume of the mucosal solution was 30 mL. The serosal solution contained 1.0 mL of the same buffer without peptides. After incubation for 0, 5, 10, 20, and 60 minutes at 37° C, the copper concentration translated to serosal fluid from mucosal fluid was determined by measuring their copper concentrations.

Result

The purification of the copper binding peptide from the mushroom, *G. frondosa,* was performed with gel filtration and ion exchange column chromatography. The elution pattern of the first gel filtration on a Sephadex G-50 column is shown in *Figure 1.* From the four copper-containing peaks observed, the fraction indicated by the bar was eluted as the major peak between the high molecular weight and the salt peak. This fraction was further purified by the DEAE-Cellulose chromatography. *Table 1* shows the results of the purification of the copper binding peptide. The copper content per mg protein of the peptide eluted by rechromatography on the Sephadex G-50 column increased up to 221 times that of the supernatant.

When the purified copper binding peptide was applied to the GS-320 HPLC column, as shown in *Figure 2,* it was revealed to be homogeneous, as it was eluted in a single symmetrical peak.

The apparent molecular weight of the copper bind-

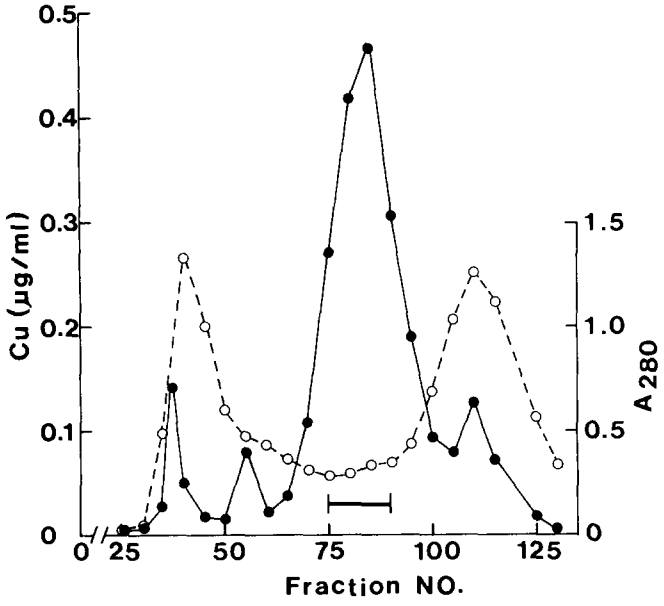

Figure 1 Gel filtration pattern of 12,000*g* supernatant of homogenate. The supernatant (150 mL) was applied to a Sephadex G-50 column (6 × 105 cm) equilibrated with 20 mmol/L Tris-HCl buffer (pH 8.0) and eluted with the same buffer at the flow rate of 86 mL/hr. Fraction size was 21.5 mL. The copper content (●—●) and absorbance at 280 nm (○---○) were measured. The low molecular weight fractions containing copper, indicated by the bar, were pooled and subjected to further purification.

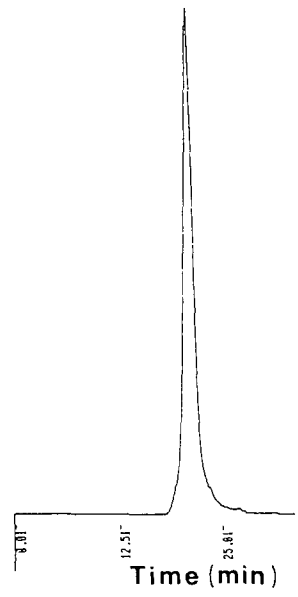

Figure 2 HPLC profile of the purified copper binding peptide. Approximately 50 μg of the copper binding peptide was applied to an Asahipak GS-320 column and eluted with 50 mmol/L ammonium acetate (pH 8.0) at the flow rate of 0.5 mL/min. Detection was 220 nm.

ing peptide was estimated to be 2,240 by gel filtration on the Sephadex G-50 column *(Figure 3)*.

The amino acid composition of the copper binding peptide is presented in *Table 2*. The peptide contains relatively high quantities of acidic amino acids (Glu, Asp), glycine and serine, which occupy about 84% of the total residues, while sulphur-containing, basic, or aromatic amino acids were rarely detected. The existence of phosphoserine observed in caseinphosphopeptide (CPP) was not confirmed by amino acid or atomic analyses. The peptide is composed of 23 residues of amino acid in accordance with the value estimated by the determination of amino groups with TNBS. Furthermore, the molecular weight of 2,240 estimated from gel filtration *(Figure 3)* agrees with that from amino acid composition. Accordingly, the copper content in the purified copper binding peptide is 1.01 mol per peptide molecule.

Table 1 Protein and copper contents in the copper-binding fraction at each purification step

Fraction	Protein[a] (mg)	Cu[a] (μg)	Cu/protein (μg/mg)
Supernatant	3740	469	0.13
Sephadex G-50	155	279	1.80
DE-32	45	193	4.29
Sephadex G-50	4.9	141	28.78

[a]From 300 g wet wt. of the fruit bodies.

As shown in *Figure 4*, in the absence of the copper binding peptide copper is mostly precipitated in the mixture of 2.5 mmol/L copper and above 5 mmol/L phosphate buffer (pH 7.4). However, when copper and phosphate are mixed in the presence of the peptide, the amount of soluble copper apparently increases because 87, 79, 45, and 29% of copper are solubilized

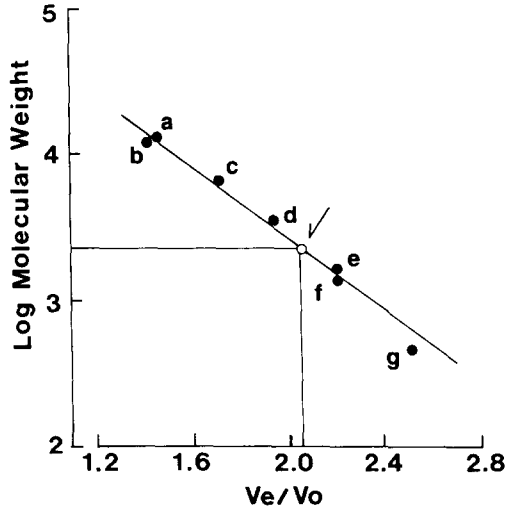

Figure 3 Molecular weight estimation of the copper binding peptide by gel filtration. The purified copper binding peptide was applied to a Sephadex G-50 column (1.0 × 50 cm) equilibrated 50 mmol/L Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and eluted with the same buffer. (a) RNase A (Mr 13500), (b) Cytochrome C (Mr 12200), (c) Aprotinin (Mr 6500), (d) B chain of insulin (Mr 3500), (e) Neurotensin (Mr 1670), (f) Bacitracin (Mr 1450), (g) Gly-Gly-Tyr-Arg (Mr 450) were used as standard protein and peptide markers. The eluted copper binding peptide is represented by the arrow.

Table 2 Amino acid composition of the copper-binding peptide

Amino acid	Composition (mol%)	Nearest integer[a]
Asp	28.8	7
Glu	15.4	4
Gly	20.2	5
Ser	19.4	4
Ala	6.0	1
Thr	5.7	1
Pro	4.4	1
Total	99.9	23

[a]The nearest integer was calculated on the assumption that proline was 1.0.

in the 5, 10, 20, and 40 mmol/L phosphate buffer, respectively. Moreover, *Figure 5* shows that the enhancing effect of the copper binding peptide on copper solubilization is dependent on the concentration of the peptide. These results demonstrate that the copper binding peptide prevents copper ions from precipitating.

The effect of pH on the stability of the copper-peptide complex was investigated with continual equilibrium dialysis against several buffers. The peptide had the highest affinity for copper at pH 7.0–8.0, while it could hardly be bound to copper under pH 5.0, as shown in *Figure 6*. The maximal copper content bound to the peptide was 1.2 mol per peptide molecule at pH 7.8.

Using the in vitro everted intestinal sac procedure, it was observed that the copper binding peptide significantly increased the transport of copper across the ileal segment of rats compared with casein or casein digestive peptides *(Figure 7)*.

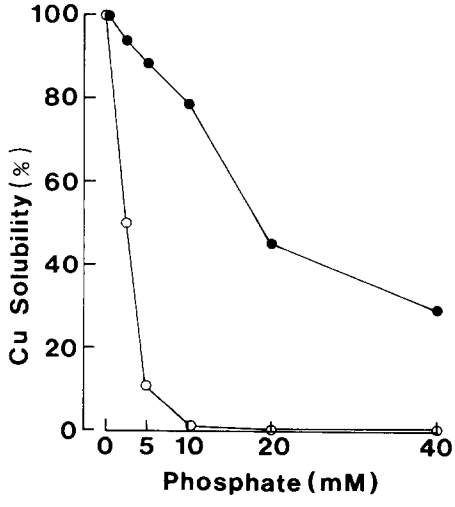

Figure 4 Effect of the copper binding peptide on copper solubility in phosphate buffers of variable phosphate concentration. Reaction mixture consisted of 2.5 mmol/L $CuSO_4$, Na-phosphate buffer (pH 7.0) with concentration of 0–40 mmol/L containing either 0.5 mg of the copper binding peptide (●—●) or not (○—○). After 2 hours, the soluble copper content was estimated.

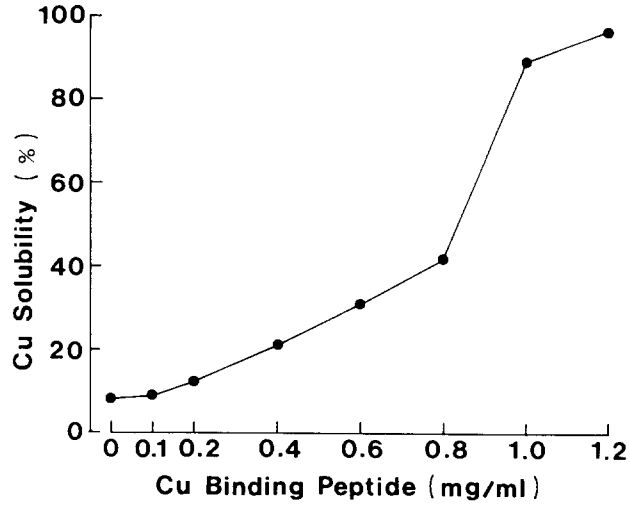

Figure 5 Effect of peptide concentration on copper solubility in a buffer with constant phosphate concentration. Under the condition of 2.5 mmol/L $CuSO_4$ and 5 mmol/L Na-phosphate buffer (pH 7.0), the soluble copper content was measured in the presence of the copper binding peptide with concentration of 0–1.2 mg/mL (●—●).

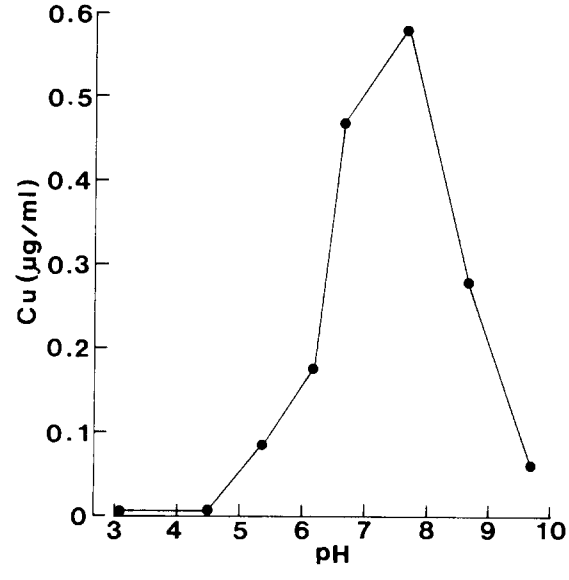

Figure 6 Effect of pH on the stability of the copper-peptide complex. Equilibrium dialysis was carried out in cellulose tubes using continual equilibrium dialyzing cell against different 20 mmol/L buffers as described in Materials and methods. After dialysis for 20 hours, the copper content of the inner solution was determined by atomic absorption spectrophotometry. The closed circle (●—●) represented the copper content that bound to the peptide by the addition of copper ions at the respective pH. Buffer systems: pH 3; KCl-HCl, pH 4–6; Sodium-acetate, pH 7–9; Tris-HCl, pH 10; NaOH-glycine.

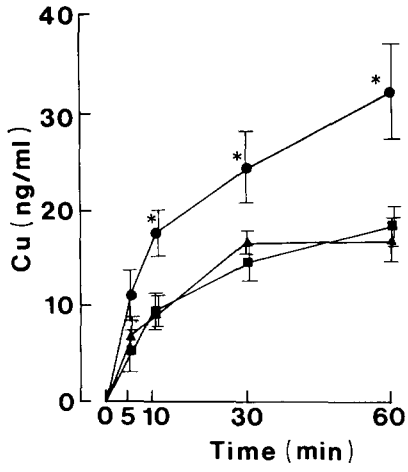

Figure 7 Effect of the copper binding peptide on the in vitro ileal transport of copper in rats. The everted sac was prepared by the method of Barr and Riegelman.[16] The copper concentration translated to serosal fluid from mucosal fluid was determined by measuring copper concentrations of both fluids using atomic absorption spectrophotometry. The Y-axis represented the copper concentration increased in serosal fluid. Each point is the mean ± SEM of seven (copper binding peptide; ●—● and casein; ▲—▲ groups) or three (casein peptides group; ■—■) intestinal sacs. *Significantly different from casein group at $P < 0.05$.

Discussion

As for the dietary factors influencing copper absorption in the intestinal lumen, it has been reported that zinc,[17,18] thiomolybdate,[19] ascorbic acid,[20] fructose,[21] and fiber[22] inhibited copper absorption, conversely amino acids,[5,23] nitrilotriacetate,[24] EDTA,[5] and phytic acid[25] enhanced its absorption. Particularly, Kirchgessner and Grassman found that copper-amino acid complexes were absorbed to a greater extent than $CuSO_4$.[5] In another study, it was reported that copper-histidine complexes did not improve copper absorption.[23] High protein diets have generally been shown to exert a positive effect on copper absorption,[2,26] but not always.[27]

Accordingly, it is assumed that copper absorption seems to be greatly influenced by the direct interaction between copper and amino acids or macropeptides formed during the luminal digestion of dietary protein. The uptake of copper by the small intestinal tract may depend on the degree of binding strength or solubilization of copper-amino acid or peptide complexes. Judging from the process of protein digestion in the small intestine, the copper absorption should be influenced mainly by peptides rather than free amino acids. As ligands of copper, however, dietary peptides regulating copper absorption have not yet been found.

In the present investigation we demonstrated that the copper binding peptide extracted and purified from the mushroom *G. frondosa* was an acidic peptide of MW 2,240 that consists of 23 amino acid residues. The peptide binds specifically to about 1 mol of copper per peptide molecule at pH 7.0–8.0. We confirmed that this peptide significantly increased the transfer of copper into the mucosal tissue. Although the present result of intestinal everted sac experiments in vitro *(Figure 7)* is insufficient to prove the increasing effect of the copper binding peptide for copper absorption, it is certain that the solubility of intra-intestinal copper is maintained by the existence of the copper binding peptide. As a result, luminal copper ions can be absorbed into mucosal cells with high efficiency by a passive type transport. It is supported by the fact that we confirmed in in vitro studies that the amount of soluble copper was obviously increased when a mixture of the peptide and copper ions was added to a solution containing phosphate, though copper generally is precipitated and changes into an insoluble state in the physiological environment *(Figures 4 and 5)*.

CPP,[28,29] phosvitin,[30] and glutathione[7] have been known to influence calcium or iron absorption. Especially, Naito et al. definitely demonstrated the in vivo formation of CPP, solubilization of calcium in the small intestinal lumen, or enhancement of calcium absorption in the presence of CPP.[9] They also suggested through the structural research that phosphoserine residues of CPP molecules might contribute to the solubilization of calcium.[31]

When the relationship between copper solubilization and absorption by the copper binding peptide is argued, the structural characteristics of the copper binding peptide come into question. Certainly the solubilization of a mineral in the lumen should be the first step in promoting the mineral bioavailability before it can be absorbed. But the increase of the soluble copper in the small intestinal lumen may not necessarily enhance copper absorption. Because, when the ligand binding force is very strong, copper absorption might be inhibited even if copper is dissolved. In fact Sato et al. showed that phosphopeptides derived from luminal digestion of the iron binding egg yolk protein inhibited iron absorption due to its tight binding to iron.[30] However, our results show that the binding force of the copper binding peptide is not so strong as to inhibit copper absorption. Therefore copper solubilization by the peptide appears to be very significant for the enhancement of copper absorption.

As shown in *Table 2*, the copper binding peptide does not contain cysteine and histidine, which have been proved to possess a high affinity for copper, and phosphoserine, which is observed in CPP and phosvitin. But this peptide is characterized by four amino acid residues, namely Asp, Glu, Ser, and Gly, which account for about 84% of total residues. Therefore, we speculate that these four amino acids may be concerned with copper solubility and binding, though further detailed investigation on primary structure of the peptide is required to elucidate the copper binding site.

Recently, a nonphosphorylated compound, synthetic polyglutamate was approved to have a soluble effect to calcium phosphate similar to CPP.[32,33] The extremely anionic property of this polypeptide may be applicable to the recognition of character of the copper binding peptide except for the difference of binding metal.

Investigation of the direct effect of peptides on various mineral absorption gives very significant information showing that copper absorption is greatly influenced by the copper binding peptide that we reported in this paper. Further studies are being undertaken in vivo and in vitro to investigate the mechanism of copper absorption enhanced with copper binding peptide.

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