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Analytical Methods

Degradation and antioxidant activities of peptides and zinc–peptide complexes during *in vitro* gastrointestinal digestion

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ABSTRACT

The degradation characteristics of three peptides (Ser-Met, Asn-Cys-Ser, and glutathione) and their zinc–peptide complexes were studied using a two-stage *in vitro* digestion model. Enzyme-resistant peptides and zinc–peptide complexes, antioxidant activities, and free amino acids released by digestive enzymes, were measured in this study. The results revealed that the three peptides and their zinc–peptide complexes were resistant to pepsin but not to pancreatin. Pancreatin can partly hydrolyse both peptides and zinc–peptide complexes, but more than half of them remaining in their original form after gastrointestinal digestion. The coordination of zinc improved the enzymatic resistance of the peptide due to lower solubility of complexes and affected the hydrolytic site of pepsin and pancreatin. Zinc–Asn-Cys-Ser, which is highly resistant to enzymatic hydrolysis and maintains Zn in a soluble form, may have potential to improve Zn bioavailability.

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1. Introduction

Zinc (Zn), a trace element that is essential for health, is the second most abundant inorganic micronutrient in the body (Cummings & Kovacic, 2009; Hambidge & Krebs, 2007). Several conditions, including growths retardation, hypogonadism, impaired immune system, and neurological dysfunctions, are attributed to Zn deficiency (Maret & Krezel, 2007). Zn deficiency appears to be common in developing countries, particularly in infants, pre-schoolers, pregnant and lactating women, and older adults (Maret & Sandstead, 2006; Wegmüller, Tay, Zeder, Brnić, & Hurrell, 2014). Intestinal Zn absorption depends on the amount of mineral consumed and its bioavailability. Supplemental Zn exists in several forms including mineral salts, metal-chelating agents, and zinc-binding proteins or peptides (Hurrell, 2002). However, mineral salts have poor bioavailability due to the inhibitory effects of dietary components such as tannins, phytate, and dietary fibre (Fredlund, Isaksson, Rossander-Hulthén, Almgren, & Sandberg, 2006; Sandberg, 2002). On the other hand, soluble, low-molecular weight organic compound, such as some amino acids, polypeptides and organic acids, can act as Zn-binding ligands and improve the bioavailability of Zn (Harzer & Kauer, 1982; Miquel & Farré, 2007).

Researchers reported that metal-chelating peptides increase mineral bioavailability either by maintaining the mineral in a soluble form or by increasing its absorption through carrier-mediated processes (Chaud et al., 2002; Miquel & Farré, 2007). The positive effects of these peptides on the absorption of iron (Fe) (Pérès et al., 1999) and Zn (Miquel & Farré, 2007) have been reported both *in vivo* and *in vitro*. Wang, Zhou, Tong, and Mao (2010) reported that yak milk casein hydrolysate (YCH) could bind with Zn ions and form complexes making Zn more soluble under simulated intestinal conditions, and YCH–Zn complexes may have potential to improve Zn bioavailability. The findings of Pérès et al. (1999) revealed that Fe bound to β-CN (1–25), a peptide obtained from the enzymatic hydrolysis of β-casein, improves Fe bioavailability in rats. However, an *in vitro* digestion assay cannot release dialyzable Fe from Fe-β-CN (1–25) complexes (Aît-Oukhatar et al., 2000). It has been reported that Fe bound to β-CN (1–25) may have a different intestinal absorption mechanism to that of Fe salts, e.g., Fe bound to casein phosphopeptides is absorbed mainly by endocytosis (Pérès et al., 1999).

Preformed Zn complexes may prevent Zn precipitation and minimise interactions with other metals and mineral inhibitors. Peptides are considered to be effective Zn binding ligands especially those isolated from protein hydrolysates, e.g., chickpea protein (Torres-Fuentes, Alaiz, & Vioque, 2011), casein (Harzer &

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Kauer, 1982; Wang et al., 2010) and sesame protein (Wang, Li, & Ao, 2012). However, the structures of these peptides, which contain metal-binding loops require further characterisation. For enhanced mineral bioavailability, it is crucial that metal-chelating peptides should be partially or fully resistant to the hydrolytic action of digestive enzymes (Miquel & Farré, 2007). However, few studies have focused on the enzymatic resistance of peptides and Zn–peptide complexes.

Sesame (*Sesamum indicum* L.), which grows widely in mainland China, is an important oilseed crop. Sesame cake, which is an inexpensive oil byproduct, is extensively used as animal feed and fertilizer. Sesame protein is rich in Glu, Asp, Arg, and Leu (Johnson, Suleiman, & Lusas, 1979). Several studies have reported that sesame protein hydrolysate (SPH) has antihypertensive properties (Nakano et al., 2006). Our previous study revealed that SPH has both zinc chelating abilities and antioxidant properties (Wang et al., 2012). In that study, six Zn chelating peptides were isolated from SPH. In this study, two of these metal-chelating peptides, which have high Zn chelating and antioxidant properties, i.e., Ser-Met (SM) and Asn-Cys-Ser (NCS), were used as models for investigating the degradation of peptide ligands and their zinc complexes in the gastrointestinal tract. Reduced glutathione (GSH) constitutes another zinc ligand with powerful antioxidant and metal chelating properties (Zhao, Ruan, & Li, 2011). Therefore, the objective of this study were to (a) investigate the resistance of peptides and Zn–peptide complexes to *in vitro* digestion conditions and (b) evaluate whether the coordination of Zn affects the resistance of these peptides to enzymatic hydrolysis.

2. Materials and methods

2.1. Materials and reagents

SM (Ser-Met) and NCS (Asn-Cys-Ser) were synthesized by Apeptide Co (Shanghai, China). The purity of the synthesized peptides was more than 95%. Enzymes (pepsin and pancreatin), chemicals and reagents, including L-glutathione reduced, triethylamine, Trolox, fluorescein sodium salt, dithizone and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), were purchased from Sigma–Aldrich (St. Louis, MO, USA), Other chemicals and reagents, which were analytical grade, were purchased from Beijing Chemical Co. (Beijing, China).

2.2. Synthesis of Zn–peptide complexes and identification by ESI-MS

The Zn–peptide complexes were prepared by mixing peptide with $ZnSO_4$ in solution. $ZnSO_4 \cdot 7H_2O$ (50.6 mg in 6 ml 50% ethanol) was added drop-wise to the peptide solution (5 ml, 50% ethanol, final concentration 0.016 mol/l) before 100 μl triethylamine was also added. After constant stirring for 1 h at room temperature, the solution was centrifuged at 8000g for 15 min. The resulting precipitate was repeatedly washed with 80% ethanol and freeze-dried for 24 h. The Zn chelating properties of SM, NCS, and GSH were evaluated by EDTA complexometric titration. The Zn–peptide complexes were purified with dithizone and ninhydrin.

Zn–SM and Zn–NCS were identified by ESI-MS (microTOF-Q II, Bruker Daltonics Inc.) with the following conditions, an ESI voltage of 3.5 kV, a nebulizer of 7.25 psi, a N_2 flow rate of 5.01 l/min, a temperature of 350 °C, a mass range of 50–1000, and a positive full ion monitoring mode.

2.3. In vitro digestion

Peptides and Zn–peptide complexes were enzymatically digested with pepsin and pancreatin according to the method of Alting, Meijer, and Van-Beresteijn (1997) with a slight modification (Ruiz, Ramos, & Recio, 2004). The samples were diluted to 10 mg/ml with 0.01 mol/l HCl (pH 2.0). Pepsin (enzyme to substrate ratio 1:50, w/w) was added and the mixture was incubated in a shaking platform for 2 h at 37 °C. The pH was first adjusted to 5.3 with 0.9 mol/l $NaHCO_3$ and subsequently to pH 7.5 with 2 mol/l NaOH. Pancreatin (enzyme to substrate ratio 1:25, w/w) was added and the mixture was incubated in a water bath for 4 h at 37 °C under constant stirring.

To investigate the changes in the peptides and zinc–peptide complexes during the *in vitro* digestion, aliquots of the digests were removed at 0, 0.5, 1, 2 (hydrolysis with pepsin), 4, and 6 h (hydrolysis with pancreatin). To terminate the enzymatic digestion, the aliquots were submerged in boiling water for 10 min and allowed to cool to room temperature. The digests were centrifuged at 8000*g* for 10 min. The supernatants (containing peptides and Zn–peptide complexes) were collected, adjusted to pH 7, and stored at −20 °C.

2.4. Content analysis of the digests

2.4.1. Content analysis of enzyme-resistant peptides and Zn–peptide complexes

Enzyme-resistant peptides and Zn–peptide complexes were analysed in SHIMADZU LC-15C equipped with an HPLC column (ZORBAX SB-C18, 4.6 mm i.d. × 250 mm, 5 μm, Agilent Technologies, USA). A gradient elution was performed with A (0.01% formic acid in water) and B (acetonitrile) at 0.3 ml/min: 5% B, 0–5 min; 60% B, 25 min; and 5% B, 40 min. The sample (20 μl) was monitored at 215 nm.

2.4.2. Determination of free amino groups

The concentration of free amino groups was determined by the TNBS method as reported by Xie, Wang, Ao, and Li (2013). Digests (10 μl) were mixed with 100 μl of potassium borate (0.1 mol/l) and 40 μl of TNBS (1.2 mg/ml) in a 96-well plate and incubated in the dark for 1 h at 37 °C. Absorbance was measured at 405 nm in a micro plate reader (Thermo Multiskan MK3). Different concentrations of glycine (0–1 μmol/l) were used to generate the standard curve. The contents of free amino groups in the digest samples were expressed as glycine amino equivalents (μmol/l), based on the equation of glycine standard curve generated.

2.4.3. Free amino acid analysis

The free amino acids in the digests were analysed by HPLC according to the method described by Vasanits and Molnár-Perl (1999) with some modifications. A pre-column derivatization method (phenylthiocarbamyl, PITC) was used. Amino acid standard solutions were used as standard and the contents of free amino acids were expressed as concentration with mg/ml.

2.5. Antioxidant activity assays

2.5.1. $ABTS^{\cdot+}$ radical scavenging assay

The $ABTS^{\cdot+}$ radical scavenging activities of the digests were assessed using the method reported by Wang and Xiong (2005) and You, Zhao, Regenstein, and Ren (2010). An $ABTS^{\cdot+}$ stock solution (2.45 mmol/l potassium persulfate and 7 mmol/l $ABTS^{\cdot+}$) was incubated in the dark for 14 h at room temperature and was diluted with 0.2 mol/l PBS (pH 7.4) to achieve an absorbance of 0.70 ± 0.02 at 734 nm. Trolox was used for the generation of the standard curve. Trolox (40 μl) of different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mmol/l) was added to 4 ml diluted $ABTS^{\cdot+}$ solution, mixed for 30 s, and allowed to stand in the dark for 6 min. Digests (40 μl of 5.0 mg/ml) were mixed with 4 ml diluted $ABTS^{\cdot+}$ solution. The absorbance of the Trolox standards and digests was

measured at 734 nm. ABTS$^{\cdot+}$ radical scavenging activity was expressed as Trolox equivalent antioxidant capacity (TEAC, g/l).

2.5.2. ORAC assay

The oxygen radical absorbance capacity (ORAC) assay was performed using fluorescein (FL) as the probe (Dávalos, Gómez-Cordovés, & Bartolomé, 2004). Black 96-well microplates and a Tecan Infinite M200 microplate reader equipped with Tecan i-Control software were used. Fluorescence measurements were performed at 37 °C. The excitation and emission wavelengths were 485 nm and 535 nm, respectively. A fluorescein stock solution (1.17 mmol/l) was prepared with 75 mmol/l phosphate buffer (pH 7.4) and stored at 4 °C for 4 weeks. The fluorescein working solution, AAPH, and Trolox solution were prepared fresh each day. The digests (20 μl) and fluorescein working solutions (120 μl of 117 nmol/l) were added to each well of the 96-well microplate, which was incubated for 12 min at 37 °C. AAPH solution (60 μl of 40 mmol/l) was then added rapidly to each well using multichannel pipet. The 96-well plate was transferred to the microplate reader, automatically shaken for 2 s and measured every minute for 120 min. The blank contained phosphate buffer instead of digest. Six calibration solutions using Trolox standards (0, 10, 30, 40, 70, and 80 μmol/l) were used.

Fluorescence measurements were normalised to the blank curve. From the normalised curves, the area under the fluorescence decay curve (AUC) was calculated by the following equation,

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=120} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The net AUC corresponding to the digest was calculated by the following equation,

$$\text{net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}}$$

The regression equations between net AUC and trolox standard were determined; the ORAC values of the digests were expressed as μmol/l trolox equivalents (μmol/l TE).

2.6. Statistical analysis

All experiments were conducted in triplicate. Data were expressed as means ± standard deviation (SD). The data were analysed by ANOVA and Duncan's mew multiple range test using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Characteristics of Zn–peptide complexes

3.1.1. Zn-chelating and antioxidant abilities of SM, NCS, and GSH

EDTA complexometric titration and ABTS$^{\cdot+}$ radical scavenging activity assays were used to evaluate the Zn-chelating and antioxidant properties, respectively, of SM, NCS, and GSH. The Zn-chelating and antioxidant properties of the peptide are shown in Table 1.

As an organic ammonia carboxy complexing agent, EDTA can chelate metal ions in a 1:1 ratio. EDTA is highly stable because of its multiple five-membered rings; therefore, EDTA is commonly used as a standard solution for ligand titration. According to the results, NCS had the highest Zn-chelating ability (60.77%), followed by GSH (40.08%), and SM (30.10%). These results were in agreements with our previous findings (Wang et al., 2012). In terms of ABTS$^{\cdot+}$ radical scavenging activity, GSH had the highest antioxidant property, followed by NCS and SM. Several peptides are multi-functional with both metal-chelating and antioxidant properties. Metals are capable of producing reactive oxygen species, that damage biomolecules. Wang et al. (2009) reported an excessive amount of Zn ions induces oxidative stress in rapeseed (*Brassica napus*) seedlings. Chelating peptides may also exhibit antioxidant activities not only by preventing this pro-oxidant effect (Torres-Fuentes et al., 2011) but also by exhibiting free radical-scavenging abilities.

Table 1
Zinc chelating and antioxidant abilities of SM, NCS and GSH.

Peptides	Zinc chelating abilities (%)	ABTS$^{\cdot+}$ radical scavenging activity (TE g/l)
SM	30.10 ± 0.02	0.02 ± 0.006
NCS	60.77 ± 0.00	0.55 ± 0.012
GSH	40.08 ± 0.03	1.03 ± 0.142

3.1.2. Mass spectra of Zn–SM and Zn–NCS

The COO^- of the peptide backbone, and the amino (NH) and carbonyl (CO) groups of the peptide bond play an important role in the formation of metal ion complexes (Katsoulakou et al., 2009). The hydroxyl group of Ser and the sulfhydryl group of Cys in SM and NCS may participate in the coordination between peptides and Zn (Wang et al., 2012).

The complexes between Zn and SM (Zn–SM) and between Zn and NCS (Zn–NCS) were identified by electrospray ionisation-mass spectrometry. Fig. 1 shows that Zn–SM and Zn–NCS were synthesized at a 1:1 ratio. The mass spectrum of Zn–SM revealed that H_2O was involved in the coordination between Zn and SM. The MS spectrum of the charged ion with m/z at 337 was $[SM - 2H_2O - Zn]^+$; the charged ions with m/z at 237 and 259.1 were $[SM + H]^+$ and $[SM - Na]^+$, respectively. Similarly, $[NCS - Zn + H]^+$, $[NCS + H]^+$, and $[NCS - Na]^+$ were in the MS spectrum of Zn–NCS.

3.2. Stability of peptides and Zn–peptide complexes during in vitro digestion

To evaluate and compare the resistance of the peptides and Zn–peptide complexes to digestive enzymes a two-stage enzymatic hydrolysis was performed. Pepsin was used to simulate gastric digestion, whereas pancreatin was used to simulate intestinal conditions. The digests were analysed by HPLC. The amount of intact peptides, Zn–peptide complexes, and free amino acids released during the *in vitro* digestion were used to assess the enzymatic resistance of the peptides and Zn–peptide complexes.

3.2.1. HPLC profile of peptides and Zn–peptide complexes

The results revealed that there was a significant difference between peptides and Zn–peptide complexes in terms of their solubility. Peptides of SM, NCS, and GSH were readily soluble in both gastric and intestinal digests whereas Zn–peptide complexes were extremely difficult to dissolve in such environment. According to the HPLC results, the solubility of Zn–NCS was higher than that of Zn–SM and Zn–GSH. The soluble portion of Zn–NCS in the digestive supernatant can be detected by HPLC, but those of Zn–SM and Zn–GSH are difficult to detect given the same conditions.

The SM, GSH, NCS, and Zn–NCS digests were separated and detected by HPLC. Fig. 2 shows the HPLC profiles before digestion and after gastric (2 h) and intestinal digestion (4 h). The results revealed that NCS and GSH were more stable during *in vitro* digestion than SM and Zn–NCS. SM and Zn–NCS were more likely to be partially degraded, especially by pancreatin.

3.2.2. Concentrations of peptides and Zn–peptide complexes during in vitro digestion

Fig. 3(A) shows the changes in SM, NCS, GSH, and Zn–NCS concentrations during the *in vitro* GI digestion. Both Zn–SM and Zn–GSH were not included in the results due to their poor solubility.

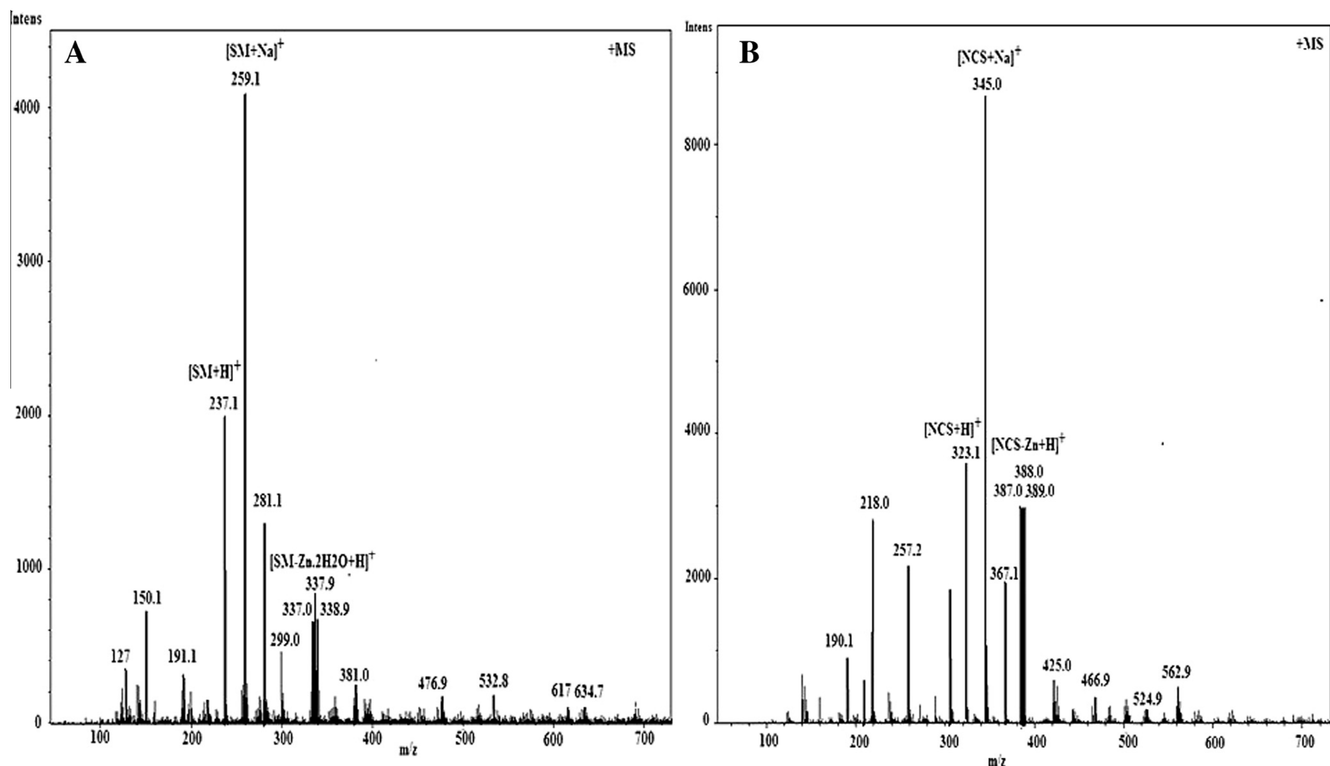

Fig. 1. The mass spectrum of Zn–SM (A) and Zn–NCS (B).

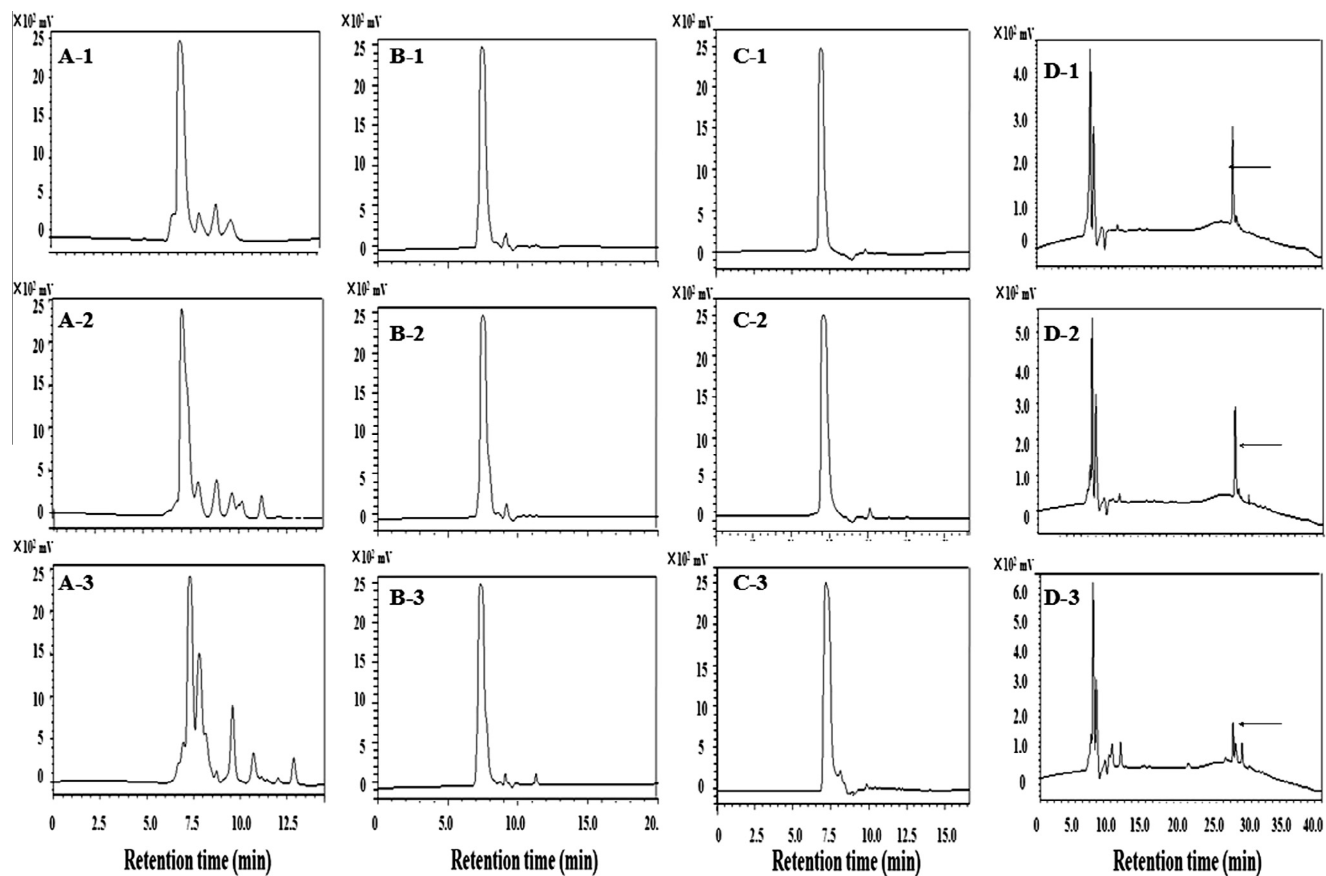

Fig. 2. RP-HPLC profiles of peptides and Zn–peptide complexes in digests. (A) SM, (B) NCS, (C) GSH, (D) Zn–NCS, and 1–3 on behalf of before digestion (0 h), after gastric digestion (2 h), and after sequential intestinal digestion (6 h), respectively.

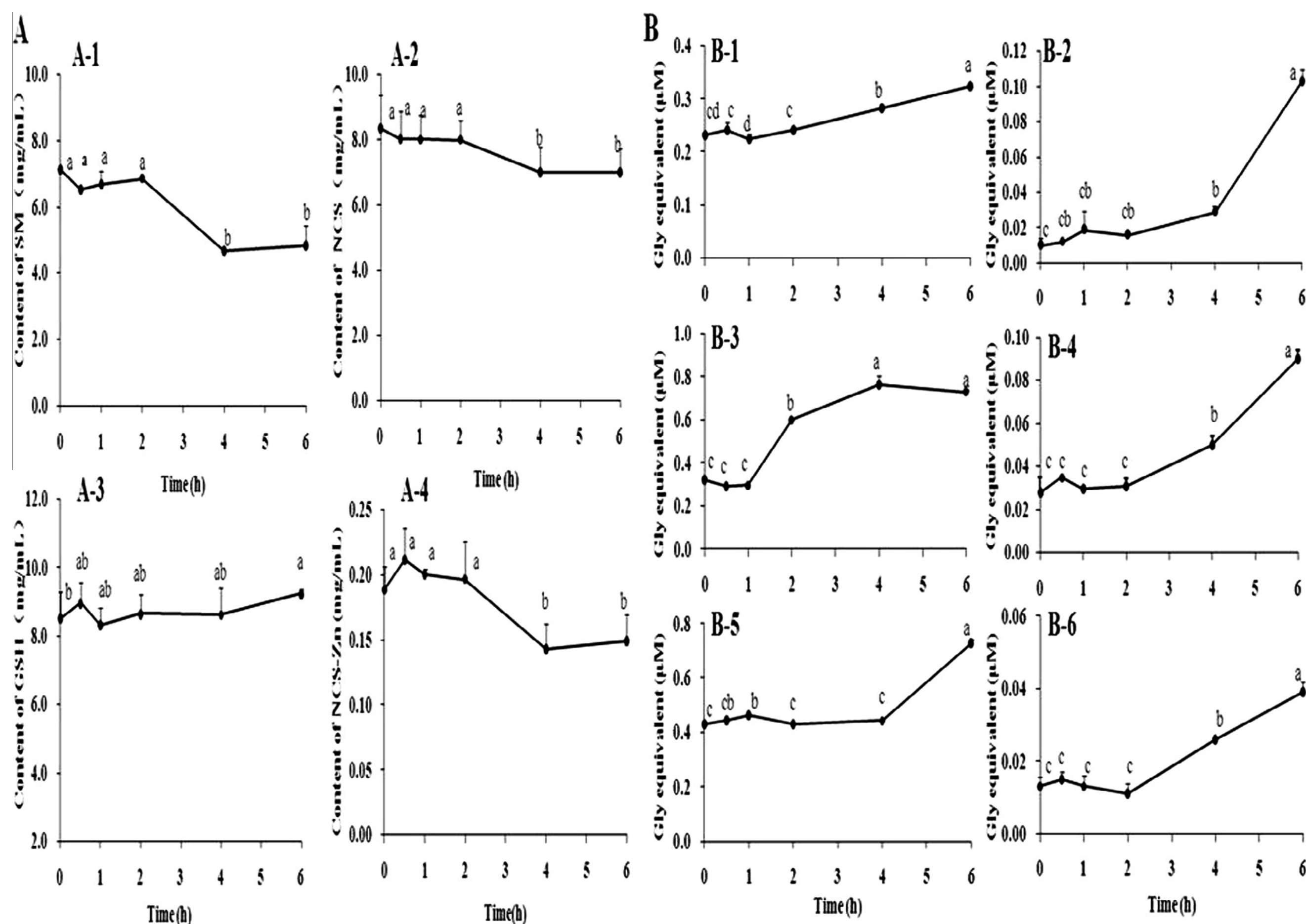

Fig. 3. (A) Concentration changes of SM (A-1), NCS (A-2), GSH (A-3) and Zn–NCS (A-4) during *in vitro* digestion. (B) Amount of amino groups of peptides and their Zn–peptide complexes during *in vitro* digestion. (B-1) SM, (B-2) Zn–SM, (B-3) NCS, (B-4) Zn–NCS, (B-5) GSH, (B-6) Zn–GSH, respectively. The data with different lowercase letters in test are significantly different ($p < 0.05$).

The concentration of GSH during the entire *in vitro* digestion was relatively stable with no significant differences amongst the different digestion periods ($p > 0.05$). Similar results were obtained between NCS and SM. These two peptides resisted gastric digestion ($p > 0.05$). However, their concentrations significantly decreased during intestinal digestion ($p < 0.05$). Similar changes also occurred in Zn–NCS. Experiments indicated that Zn–NCS could survive gastric digestion because of the buffering effect of the complex.

When the Zn–peptide complexes were adjusted to pH 2.0 with HCl, precipitates formed. During the *in vitro* digestion, the pH of the digests changed from 2.0 to 6.5, which is not the optimum pH of pepsin. On the other hand, NCS, SM, and GSH were readily soluble in gastric conditions. Therefore, Zn–peptide complexes can resist the action of pepsin because of their buffering action.

The concentrations of SM, NCS, GSH, and Zn–NCS before digestion (0 h) were considered to be 100%. The survival rates after gastric (2 h) and intestinal digestion (6 h) were calculated. The survival rates of SM and NCS after gastric digestion were 96.18% and 95.69%, respectively. This finding suggests that SM and NCS were highly resistant to pepsin. However, the survival rates of all peptides and Zn–peptide complexes decreased during the intestinal digestion. SM had the highest reduction in survival rate, followed by Zn–NCS and NCS. At the end of the *in vitro* digestion, 67.84% of SM, 83.61% of NCS, and 80.62% of Zn–NCS remained intact.

The degradation of peptides contributes to the release of free amino groups during digestion. Therefore, the amount of free amino groups is an indicator of the actual number of peptide bonds cleaved by digestive enzymes (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). As Zn–SM and Zn–GSH could not be detected by HPLC because of their poor solubility, the amounts of free amino groups from peptides and Zn–peptide complexes during *in vitro* digestion were determined to reflect their stability in simulated gastrointestinal conditions.

As shown in Fig. 3(B), during gastric digestion, free amino groups in both peptides and Zn–peptide complexes increased inconspicuously, except for NCS, where the free amino groups increased twice within the 1–2 h gastric digestion. Following intestinal digestion, the free amino groups in both peptides and Zn–peptide complexes increased ($p < 0.05$).This result suggests that the peptides (i.e., SM, NCS, and GSH) and Zn–peptide complexes (i.e., Zn–SM, Zn–NCS, and Zn–GSH) were only slightly sensitive to gastric conditions, they were nevertheless digested by pancreatin. These results are consistent with the HPLC results. However, unlike in the peptide digests, only a few free amino groups were present in the Zn–peptide digests. This phenomenon may be attributed to the poor solubility of Zn–peptide complexes and the fact that the supernatant (as opposed to the precipitate) was used in the experiment. The result also suggests that only the soluble fraction of the peptides and Zn–peptide complexes can be degraded; insoluble components cannot interact with digestive enzymes. Moreover, the increase in free amino groups from the Zn–peptide complexes occurred at higher rate than that from the peptides, particularly during the intestinal digestion.

Table 2
Concentration of free amino acids in digests during GI digestion *in vitro*.

IC[d]	NCS (mg/ml) 8.35 ± 1.02		Zn–NCS (mg/ml) 0.18 ± 0.02		GSH (mg/ml) 8.52 ± 0.76		Zn–GSH (mg/ml) –[i]	
	Cys	Ser	Cys	Ser	Cys	Gly	Cys	Gly
IC[d]	0.015 ± 0.000[c]	0.312 ± 0.007[c]	–	0.016 ± 0.000[c]	–	0.141 ± 0.000[b]	0.014 ± 0.000[b]	0.019 ± 0.000[a]
CG[e]	0.032 ± 0.001[b]	0.416 ± 0.049[b]	–	0.031 ± 0.003[b]	–	0.195 ± 0.031[b]	0.014 ± 0.000[b]	0.016 ± 0.000[a]
CI[f]	0.346 ± 0.002[a]	1.187 ± 0.025[a]	0.007 ± 0.000	0.112 ± 0.003[a]	0.390 ± 0.036	3.396 ± 0.105[a]	0.023 ± 0.001[a]	0.021 ± 0.004[a]
CCG[g]/IC	0.20%	1.25%	0.00%	8.40%	0.00%	0.63%		
CCI[h]/IC	3.96%	10.48%	3.63%	53.27%	4.58%	38.20%		

[a–c] The data with different lowercase letters in test are significantly different ($p < 0.05$).
[d] IC means the initial concentration before digestion.
[e] CG means the concentration after 2 h gastric digestion.
[f] CI means the concentration after following 4 h intestinal digestion.
[g] CCG means the concentration change of amino acids after gastric digestion.
[h] CCI means the concentration change of amino acids after intestinal digestion.
[i] Undetected.

3.2.3. Free amino acids released from peptides and Zn–peptide complexes

Enzymes hydrolyse at specific cleavage sites in polypeptides chains. The free amino acids were studied to determine the cleavage sites in the peptides and Zn–peptide complexes. The concentrations of amino acids in NCS, GSH, and their Zn complexes are shown in Table 2.

In both NCS and Zn–NCS, Ser was released prior to Cys. The results are consistent with those obtained by Schmelzer et al. (2007), who reported that the C-terminal end of the peptide is easily hydrolysed by digestive enzymes. The coordination of Zn had little effect on the hydrolytic activity of pepsin in Zn–NCS. In NCS, a small amount of Cys was released from NCS; however, free Cys was not detected in Zn–NCS following gastric digestion. On the other hand, pancreatin recognised both Cys and Ser in NCS and Zn–NCS, and free Cys and Ser increased after intestinal digestion. These results suggest that both NCS and Zn–NCS can be partially hydrolysed.

On the other hand, GSH and Zn–GSH were stable under gastric conditions. This was evident by the fact that there were no significant differences in the concentration of free amino acids (Cys and Gly) following gastric digestion. Cys and Gly increased following the addition of pancreatin. However, the coordination of Zn had obvious effect on the hydrolytic activity of pancreatin in Zn–NCS. In GSH, Gly was released prior to Cys; while the same amount of free Cys and Gly was detected in Zn–NCS following intestinal digestion. Therefore, the coordination of Zn had a certain effect on the cleavage sites of pepsin and pancreatin.

3.3. Changes in the antioxidant activities of peptides and Zn–peptide complexes during in vitro digestion

In the presence of pepsin and pancreatin, substrates are hydrolysed into different fragments with different antioxidant activities. Therefore, the changes in antioxidant activities were used as

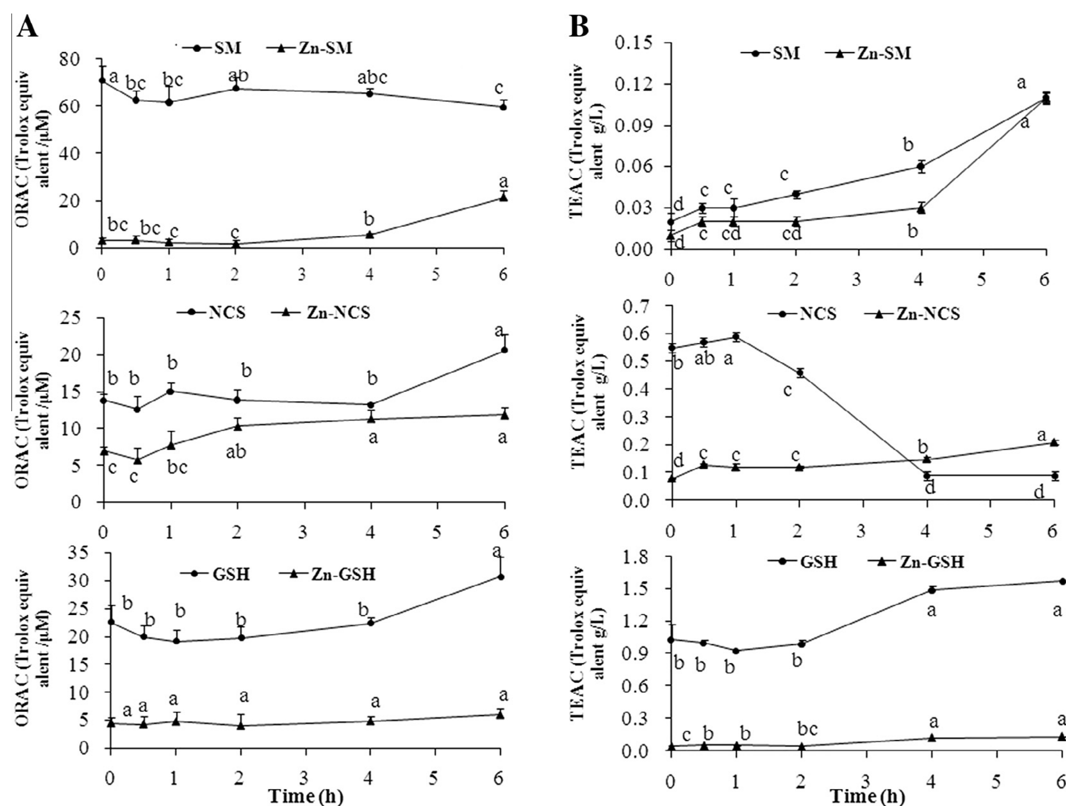

Fig. 4. Changes of ORAC values (A) and ABTS$^{·+}$ radical scavenging activity (B) of peptides and zinc–peptide complexes during *in vitro* digestion. The data with different lowercase letters in test are significantly different ($p < 0.05$).

another indicator of the enzymatic resistance of peptides and Zn–peptide complexes.

Antioxidant activity was determined by a number of different assays. TEAC and ORAC assays are commonly used for assessing the antioxidant capacity of food components (Clausen, Skibsted, & Stagsted, 2009).

The ORAC values of peptides and Zn–peptide complexes during *in vitro* digestion are shown in Fig. 4(A). The changes in NCS and GSH were similar. However, there were no significant changes in the antioxidant activity during gastric digestion and during the first 2 h of intestinal digestion ($p > 0.05$). Antioxidant activity significantly increased in the last 2 h of intestinal digestion ($p < 0.05$). The antioxidant activities of Zn–NCS and Zn–SM gradually increased during digestion; the ORAC values of these complexes during digestion were higher than those prior to digestion ($p < 0.05$). The results were more significant for Zn–SM than for Zn–NCS. In Zn–GSH, the ORAC values were stable during the entire digestion ($p > 0.05$). The ORAC values of SM declined with digestion. After digestion, the activity was 15.76% lower than that before digestion ($p < 0.05$).

Clausen et al. (2009) reported that Cys and Met are active in the ORAC assay, with Met being more active than Cys. Moreover, Cys is one of the most active amino acids in the TEAC assay. Therefore, the ORAC values of NCS, Zn–NCS, GSH, Zn–GSH, and Zn–SM increased during the *in vitro* digestion, especially during the intestinal digestion step because of the contribution of Met and Cys by the action of pancreatin.

The TEAC values of SM, Zn–SM, NCS, Zn–NCS, GSH, and Zn–GSH are shown in Fig. 4(B). The changes in the antioxidant capacity of SM and Zn–SM were similar to those obtained by the $ABTS^{\cdot+}$ radical scavenging activity assay. The TEAC values of SM increased ($p < 0.05$) after 1.5 h of gastric digestion. Although TEAC values for Zn–SM appeared to increase during gastric digestion, the results were not significant. The TEAC values of both SM and Zn–SM significantly increased during the intestinal digestion ($p < 0.05$). The antioxidant capacity of NCS and Zn–NCS were opposing; a significant decrease in NCS and a significant increase in Zn–NCS occurred during intestinal digestion ($p < 0.05$). Clearly, GSH and Zn–GSH appeared to follow a similar trend, but the changes in antioxidant capacity were not significant ($p > 0.05$). The highest antioxidant capacity was obtained during the intestinal digestion ($p < 0.05$) of GSH and Zn–GSH. The change in $ABTS^{\cdot+}$ radical scavenging activity during the *in vitro* digestion is linked to enzymatic action. An increase in antioxidant activity indicates that a fragment with strong activity was produced during digestion (Chen & Li, 2012). On the other hand, a decrease in antioxidant activity indicates that a fragment with strong activity was hydrolysed.

When comparing the antioxidant activities amongst peptides and Zn–peptide complexes, we observed that the changes were more significant in the Zn–peptide complexes, specifically during the intestinal digestion. However, there were significant changes during intestinal digestion and slight changes during gastric digestion in both peptides and Zn–peptide complexes.

4. Conclusion

Metal–peptide complexes are increasingly recognised as a source of organic trace minerals. However, few researchers have studied the effects of different peptide ligands on Zn bioavailability. In this study, an *in vitro* digestion was used to evaluate the resistance of a di-peptide (SM), tri-peptide (NCS), and their Zn–peptide complexes to simulated human digestive conditions. Additionally, the effect of different peptide ligands on Zn solubility was investigated. The solubility of Zn–NCS was higher than that of Zn–SM and Zn–GSH. The results revealed that even though peptides and Zn–peptide complexes were partially resistant to intestinal conditions, more than half of the residual peptides and Zn–peptide complexes remained in their intact forms. NCS is both an excellent antioxidant and Zn chelator. Furthermore, NCS was partially resistant to hydrolysis and can potentially increase Zn absorption and bioavailability. Therefore, the Zn–NCS complex could be an alternative to inorganic salts currently used in supplements. *In vivo* studies are needed to confirm the results.

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