

RESEARCH

Open Access



# Identification of zinc-binding peptides in ADAM17-inhibiting whey protein hydrolysates using IMAC-Zn<sup>2+</sup> coupled with shotgun peptidomics

M. Chinonye Udechukwu<sup>1</sup>, Chi Dang<sup>2</sup> and Chibuikwe C. Udenigwe<sup>2,3\*</sup> 

## Abstract

Food components possessing zinc ligands can be used to inhibit zinc-dependent enzymes. In this study, zinc-binding peptides were derived from whey protein hydrolysates, and their ultrafiltration (> 1 and < 1 kDa) fractions, produced with Esperase (WPH-Esp), Everlase and Savinase. Immobilized metal affinity chromatography (IMAC-Zn<sup>2+</sup>) increased the zinc-binding capacity of the peptide fraction (83%) when compared to WPH-Esp (23%) and its < 1 kDa fraction (40%). The increased zinc-binding capacity of the sample increased the inhibitory activity against the zinc-dependent “a disintegrin and metalloproteinase 17”. LC-MS/MS analysis using a shotgun peptidomics approach resulted in the identification of 24 peptides originating from bovine β-lactoglobulin, α-lactalbumin, serum albumin, β-casein, κ-casein, osteopontin-k, and folate receptor-α in the fraction. The identified peptides contained different combinations of the strong zinc-binding group of residues, His+Cys, Asp+Glu and Phe+Tyr, although Cys residues were absent in the sequences. In silico predictions showed that the IMAC-Zn<sup>2+</sup> peptides were non-toxins. However, the peptides possessed poor drug-like and pharmacokinetic properties; this was possibly due to their long chain lengths (5–19 residues). Taken together, this work provided an array of food peptide-based zinc ligands for further investigation of structure-function relationships and development of nutraceuticals against inflammatory and other zinc-related diseases.

**Keywords:** Zinc, Zinc ligands, Food protein, Bioactive peptides, Immobilized metal affinity chromatography, Ultrafiltration, ADAM17, Peptidomics, Bioinformatics

## Introduction

Food proteins are rich sources of peptides with an inherent capacity to bind divalent metals, such as zinc (Sun et al. 2020). Zinc is a Lewis acid and can serve as an electron pair acceptor from Lewis bases, such as amino acid ligands. Thus, the zinc-binding property of food peptides is due to their structural composition of specific amino acid residues, such as His, Cys, Asp, Glu, Phe and

Tyr, whose functional side chains act as nucleophilic ligands to form coordinate complexes with zinc (Bischoff and Schlüter 2012; Udechukwu et al. 2016; Walters et al. 2018; Sun et al. 2020). Such zinc-binding peptides could have useful nutritional applications as dietary zinc carriers to enhance zinc bioaccessibility and bioavailability (Walters et al. 2018; Udechukwu et al. 2016, 2018). Notably, our previous study have provided insight into the pharmacological relevance of zinc-binding peptides as enzyme inhibitors for controlling inflammatory diseases mediated by the pro-inflammatory actions of tumour necrosis factor (TNF)-α. Zinc-binding Cys-containing tripeptides derived from rye secalin inhibited the in vitro

\* Correspondence: [cudenigw@uottawa.ca](mailto:cudenigw@uottawa.ca)

<sup>2</sup>Department of Chemistry and Biomolecular Sciences, Faculty of Science, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada

<sup>3</sup>School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Full list of author information is available at the end of the article



© The Author(s). 2021 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

activity of ADAM17 (a disintegrin and metalloproteinase 17) (Udechukwu et al. 2017). ADAM17 is a zinc-dependent enzyme that activates pro-inflammatory TNF- $\alpha$  by the cleavage of its extracellular domain. The primary means of such enzyme inhibition is via the zinc cofactor in the catalytic site, which is crucial to structural integrity and substrate interaction.

The identification of zinc-binding food peptides would enable the understanding of their structure-function relationship and facilitate their future applications in human nutrition and health promotion. Zinc-binding peptides are usually produced from food proteins following bioassay-guided procedures, which typically begin with enzymatic hydrolysis and release of the peptide ligands from the parent proteins. The protein hydrolysates are then subjected to post-hydrolysis processing/purification steps, involving immobilized metal ( $Zn^{2+}$ ) affinity chromatography (IMAC- $Zn^{2+}$ ), in which peptides with high affinity for zinc are retained on the chromatographic column and retrieved during the elution step (Sun et al. 2020). Using IMAC, some studies derived zinc-binding peptides, including LAN, SM and NCS from sesame proteins (Wang et al. 2012); AR, GKR, EPSH and NSM from rapeseed proteins (Xie et al. 2015); a 16-mer peptide from oyster proteins (Chen et al. 2013); and 10-mer and 12-mer peptides from wheat germ proteins (Zhu et al. 2015). Other peptides, CQV and QCA, derived from rye secalin hydrolysates have also been reported to possess zinc-binding capacity (Udechukwu et al. 2017). These studies identified the peptides following purification steps and targeted mass spectrometry of selected chromatographic fractions. Hence, the peptide list represents only a glimpse of the potential of the food proteins in providing a structurally diverse range of zinc ligands. For a comprehensive identification of peptides present in protein hydrolysates, shotgun peptidomics provides an untargeted approach for peptide profiling and quantitation (Agyei, Tsopmo, and Udenigwe 2018). Therefore, the objectives of this study were to apply IMAC- $Zn^{2+}$  coupled with shotgun peptidomics to systematically produce and identify potent zinc-binding peptide motifs from whey protein hydrolysates, investigate the *in silico* pharmacokinetics and safety profiles of the peptides, and evaluate the functional significance of the IMAC- $Zn^{2+}$  on inhibitory activity of the peptide fractions against zinc-dependent ADAM17.

## Materials and methods

### Materials

Bovine whey protein isolate (WPI) powder was purchased from Bulk Barn Foods Ltd. (Truro, NS, Canada). Proteases (Esperase, Everlase and Savinase) and 8-anilino-1-naphthalene sulfonic acid (ANS) were purchased from Sigma-

Aldrich (now MilliporeSigma, Oakville, ON, Canada), and 4-(2-pyridylazo) resorcinol, sodium dodecyl sulphate (SDS), dithiothreitol (DTT) and zinc sulphate heptahydrate ( $ZnSO_4 \cdot 7H_2O$ ) were purchased from Fischer Scientific (Ottawa, ON, Canada).

### WPI hydrolysis and ultrafiltration

Aqueous suspensions of bovine WPI (5%, w/v) were hydrolyzed with Esperase, Everlase or Savinase for 5 h under different optimal conditions (60 °C, pH 8.0 for Esperase and Everlase; 55 °C, pH 8.0 for Savinase), as previously reported by our group (Udechukwu et al. 2018). Hydrolysis was terminated by adjusting the reaction mixtures to pH 4.0 using 1 M HCl, in order to inactivate the enzymes, followed by centrifugation at 10,000 $\times$ g for 10 min (Thermo Scientific Sorvall ST16R, ThermoFisher Scientific, Waltham, MA, USA). The supernatant was then adjusted to pH 7.0 using 1 M NaOH and freeze-dried to obtain the hydrolysate powders from Esperase (WPH-Esp), Everlase (WPH-Ever) and Savinase (WPH-Sav). The peptides in each of the WPH (10 mg/mL) were fractionated by passage through an ultrafiltration membrane of 1 kDa molecular weight cut-off using an Amicon Stirred Cell (EMD Millipore Corporation, Darmstadt, Germany) under nitrogen gas at 40 psi. The permeates (< 1 kDa peptide fraction) and the retentates (> 1 kDa peptide fraction) were collected after 4 h and freeze-dried at a surface temperature of 24 °C and a pressure of < 0.2 mbar (Rotary Vane Vacuum Pump, Labconco, Kansas, MI, USA) using the FreeZone 18 L Console Freeze Dry System (Labconco, Kansas, Missouri, USA), to obtain the fraction powders. The peptide fractions were then evaluated for zinc-binding capacity and the most active fraction (WPH-Esp, < 1 kDa) was chosen for the subsequent procedure.

### Immobilized metal ion affinity chromatography (IMAC)

Peptides with strong zinc-binding affinity in the < 1 kDa fraction of WPH-Esp were isolated on a 1 mL HiTrap IMAC Sepharose 6 Fast Flow prepacked column (GE Healthcare, Chicago, IL, USA). The column was loaded on a peristaltic pump (Thermo Fisher, Ottawa, ON, Canada) and washed with 5 column volume (CV) of deionized water. Thereafter, the column was charged with 0.1 M  $ZnSO_4 \cdot 7H_2O$  and unbound zinc was washed off with 5 CV of water. This process was followed by column equilibration with 5 CV of 0.02 M sodium phosphate/0.5 M NaCl buffer (pH 7.2). The WPH-Esp sample (20 mg/mL) was prepared in the phosphate buffer and passed through the column at a rate of 2.5 mL/min to bind the immobilized zinc. Thereafter, the column was washed with 10 CV of the phosphate buffer to remove unbound peptides. Bound peptides were recovered using the pH method of elution with 10 CV of sodium acetate buffer (pH 4).

The resulting fraction was desalted on a C18 Extraction Disk Cartridge (Chromatographic Specialties, Brockville, ON, Canada). The column resin was activated with 100% acetonitrile and equilibrated with the binding buffer, 0.1% trifluoroacetic acid (TFA) in water. The sample was prepared in the binding buffer and passed through the column, which was then centrifuged for 2 min at 110×g to facilitate salt removal. The column was then washed twice with the binding buffer to remove residual salt, followed by peptide elution using an aqueous solution of 0.1% TFA and 70% acetonitrile. Residual organic solvent was removed from the peptide sample using a nitrogen evaporator. The bound peptide solutions were pooled and freeze-dried to obtain a powder.

#### Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of the zinc-binding peptides

LC-MS/MS analysis was conducted on an Orbitrap analyser (Q-Exactive, ThermoFisher, San Jose, CA, USA) outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher, San Jose, CA, USA) by the Mass Spectrometry Facility at SPARC BioCentre (Toronto, ON, Canada). The IMAC peptide fraction was dissolved in 0.1% formic acid and loaded onto a PepMap RSLC EASYSprayC18 column (75 µm × 50 cm, 2 µm particles) (Thermo Fisher) at a pressure of 800 bar. Peptides were eluted over 60 min at a flowrate of 250 nl/min using a 0–35% acetonitrile gradient in 0.1% formic acid. The eluted peptides were then introduced into the Q-Exactive mass spectrometer (Thermo Fisher) by nano-electrospray. The instrument consisted of one MS full scan (400–1500 m/z) in the Orbitrap mass analyser with an automatic gain control (AGC) target of 1e6, maximum ion injection time of 120 ms and a resolution of 70,000, followed by 10 data-dependent MS/MS scans with a resolution of 17,500, an AGC target of 1e6, maximum ion time of 120 ms, and one microscan. The intensity threshold to trigger a MS/MS scan was set to 1.7e4. Fragmentation occurred in the HCD trap with normalized collision energy set to 27. The dynamic exclusion was applied using a setting of 10 s.

#### Peptidomics data analysis

Peptidomics data analysis was performed using PEAKS® software (Bioinformatic Solutions, Waterloo, ON, Canada) (Zhang et al. 2012). For a reliable peptide identification, the workflow used for mass spectra analysis included both the database search strategy and de novo sequencing (Zhang et al. 2012; Rajendran et al. 2016). The protein database of *Bos taurus* (Bovine) was retrieved from UniProtKB/Swiss-Prot database (<http://www.uniprot.org/>) and the search parameters were set at precursor mass tolerance of 10 ppm and fragment ion mass tolerance of 0.02 Da.

#### In vitro zinc-peptide binding evaluation

The whey protein hydrolysates were evaluated for zinc binding as previously reported (Jakob et al. 2000). The assay principle is based on the reaction of 4-(2-pyridylazo) resorcinol with free zinc ions in solution to form a red coordination complex (Sun et al. 2020). The peptide samples and reagents were prepared in 40 mM HEPES-KOH buffer (pH 7.5). Then, 250 µL of the samples (at final concentrations of 0.1–1 mg/mL) was mixed with 125 µL each of 8 mM DTT and 250 µM ZnSO<sub>4</sub>·7H<sub>2</sub>O. The blank experiment contained all assay components except the samples, which were substituted with an equal volume of HEPES-KOH buffer. The mixtures were incubated at 37 °C for 10 min followed by the addition of 25 µL of 2 mM 4-(2-pyridylazo) resorcinol and absorbance measurement at 500 nm. The percentage zinc-binding capacity was calculated as  $[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$ , where A is absorbance.

#### In silico ADME/Tox profile and metal-binding potential evaluation of the identified peptides

The identified peptides were subjected to further characterization using bioinformatics tools. SwissADME (<http://www.swissadme.ch/index>), a validated web-based tool, was used for predicting and evaluating the ADME (absorption, distribution, metabolism, and excretion) properties, estimating their physiochemical properties, lipophilicity, pharmacokinetics, and drug-likeness of the peptides based on Lipinski's rule-of-five (Daina et al. 2017). The 24 sequences were submitted for evaluation using their SMILES strings retrieved from BIOPEP-UWM. Additionally, ToxinPred (<https://webs.iitd.edu.in/raghava/toxinpred/index.html>) was used to predict the potential toxicity of the peptides (Gupta et al. 2013). A threshold of 0.0 (automated) was applied. The potential in vitro and in vivo stability of the peptides was also predicted using ExPASy ProtParam (<https://web.expasy.org/protparam/>), yielding the instability index and aliphatic index. Lastly, the total percentage of metal binding sites was calculated as the percentage of metal-binding residues, His+Cys, Asp+Glu and Tyr+Phe (Lu et al. 2012; Remko et al. 2011).

#### In vitro ADAM17 inhibition assay

ADAM17 inhibitory activities of WPH-Esp, the < 1 kDa and IMAC-Zn<sup>2+</sup> fractions were determined at 1 mg/mL as previously reported (Udechukwu et al. 2017). All samples (25 µL), prepared in 25 mM Tris-HCl buffer (pH 8.0), were mixed with 25 µL of 0.4 ng/µL recombinant human ADAM17 and 10 µM ZnSO<sub>4</sub>·7H<sub>2</sub>O. The mixtures were equilibrated at 37 °C for 5 min and 25 µL of 40 µM ADAM17 fluorogenic peptide substrate was then added. Fluorescence was measured at the excitation and emission wavelengths of 320 and 405 nm, respectively for 5 min at

30 s intervals. ADAM17 inhibition was calculated as  $[(R_0 - R/R_0)] \times 100$ , where  $R_0$  and  $R$  are the reaction rates in the absence and presence of the peptides, respectively.

### Statistical analysis

Apart from the LC-MS/MS analysis, experiments were conducted in triplicate and results were expressed as mean  $\pm$  standard deviation. Significant differences ( $P < 0.05$ ) between results were determined by one-way analysis of variance followed by the Holm–Sidak multiple comparison test. The statistical analyses were done using SigmaPlot 12.1 (Systat Software, San Jose, CA, USA).

## Results and discussion

### Whey protein hydrolysates demonstrated zinc-binding capacity

The classic method of producing bioactive peptides involves enzymatic hydrolysis of food proteins, which can be selected based on knowledge of the presence of bioactive peptides within their primary structures (Udenigwe and Aluko 2012). Whey proteins have not been reported to bind zinc; however, they are potential sources of zinc-binding peptides due to their capacity to bind other divalent metals, such as calcium ( $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin) and iron (lactoferrin) (Walzem et al. 2002; Krissansen 2007). The release of peptides during hydrolysis with Esperase, Everlase and Savinase is demonstrated by the higher content of free amino nitrogen of the resulting WPH (~ 200 Serine mequiv  $\text{NH}_2/\text{g}$  protein) compared to the parent WPI (65 Serine mequiv  $\text{NH}_2/\text{g}$  protein) (Udechukwu et al. 2018). Protease-induced protein hydrolysis usually yields hydrolysates with different structural properties, due to the different cleavage specificity of the proteases, which influences the molecular interactions of the peptides (Tavano 2013). For instance, our previous study showed that WPH-Esp, WPH-Ever and WPH-Sav had similar and the highest net anionic charge (negative zeta-potential) of 10 different WPH samples studied (Udechukwu et al. 2018). Consequently, the three hydrolysates demonstrated similar concentration-dependent zinc-binding capacity (Fig. 1). The anionic property provides a strong structural feature for the peptide samples to bind the cationic zinc via electrostatic interaction, which is proposed to subsequently result in the inhibition of zinc-dependent enzymes, such as ADAM17.

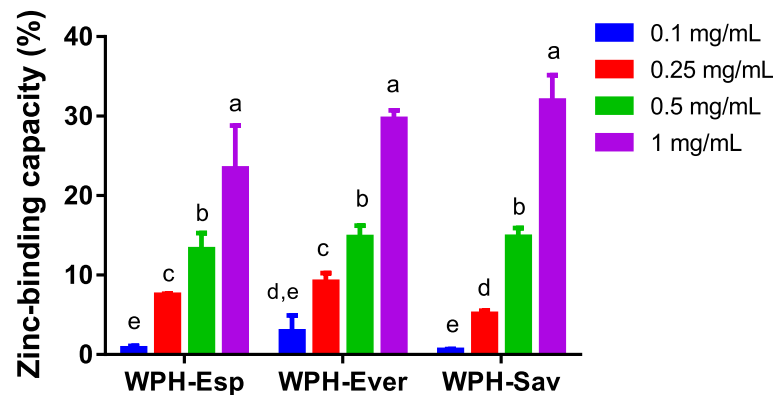
### Molecular weight-based fractionation of WPH enhanced zinc-binding capacity

Protein hydrolysis yields peptides of a broad molecular weight range. Therefore, downstream processing of bioactive peptides usually incorporates membrane ultrafiltration to isolate peptides of specific molecular sizes, depending on the intended application (Korhonen and Pihlanto 2006; Udenigwe and Aluko 2012). Higher

molecular weight peptides are preferable for functional properties, such as gelation. In contrast, smaller sized peptides are desirable for functional food application as they are more likely to withstand gut proteolysis and permeate the intestinal cells into the blood stream (Gardner 1988). The peptides in WPH-Esp, WPH-Ever and WPH-Sav were separated into higher ( $> 1$  kDa) and lower ( $< 1$  kDa) molecular weight fractions, with the aim of selecting the most promising peptide fraction. As shown in Fig. 2a–c, ultrafiltration increased the zinc-binding capacity of the WPH samples, and both the low- and high-molecular weight fractions showed concentration-dependent binding. The zinc-binding capacity of the  $> 1$ -kDa fractions was significantly higher than that of the  $< 1$ -kDa fractions for WPH-Ever (Fig. 2b) and WPH-Sav (Fig. 2c). This suggests that more zinc ligands were present in the larger peptides for these samples. Conversely, the zinc-binding sites appeared to be equally distributed between the  $> 1$ -kDa and  $< 1$ -kDa peptide fractions of WPH-Esp (Fig. 2a). This indicates that both fractions are likely to have similar interaction with the zinc cofactor of enzymes. Besides, the  $< 1$ -kDa fraction of WPH-Esp had significantly higher ( $P < 0.05$ ) zinc-binding capacity at 1 mg/mL compared to the similarly sized fractions of WPH-Ever and WPH-Sav. Therefore, the  $< 1$  kDa fraction of WPH-Esp was chosen as the best candidate for isolation and identification of the zinc-binding peptides.

### Affinity purification by IMAC- $\text{Zn}^{2+}$ enhanced zinc-binding and ADAM17 inhibitory activity of the WPH-Esp $< 1$ kDa fraction

As shown in Fig. 3a, strong metal-binding peptides can be selectively enriched from protein hydrolysates using IMAC. Besides the high selectivity, IMAC is mild and non-denaturing, making it suitable for isolation of metal-binding proteins and peptides with high sample recovery (Gaberc-Porekar and Menart 2001). As shown in Fig. 3b, zinc-binding capacity of the IMAC- $\text{Zn}^{2+}$  peptide fraction (IMAC-ZBP) was significantly higher ( $P < 0.05$ ) than that of the parent hydrolysate (WPH-Esp) and  $< 1$ -kDa ultrafiltration fraction. This demonstrates an efficient separation of the zinc-binding peptides. Peptides separate on IMAC columns based on their interaction with immobilized metal ions (Sun et al. 2020). Peptidic metal ligands, such as Asp, Glu and His, are deprotonated at neutral-basic pH conditions and hence form reversible metal complexes on the IMAC- $\text{Zn}^{2+}$  column (Block et al. 2009), which dissociate at acidic pH (Cheung, Wong, and Ng 2012). The IMAC approach has been applied in isolating peptides with high metal-binding capacity than their parent protein hydrolysates (Torres-Fuentes, Alaiz, and Vioque 2011; Chen et al. 2013; Zhu et al. 2015).



**Fig. 1** Zinc-binding capacity of whey protein hydrolysates (WPH) produced with Esperase (WPH-Esp), Everlase (WPH-Ever) and Savinase (WPH-Sav). Bars with different letters are significantly different mean values ( $P < 0.05$ )

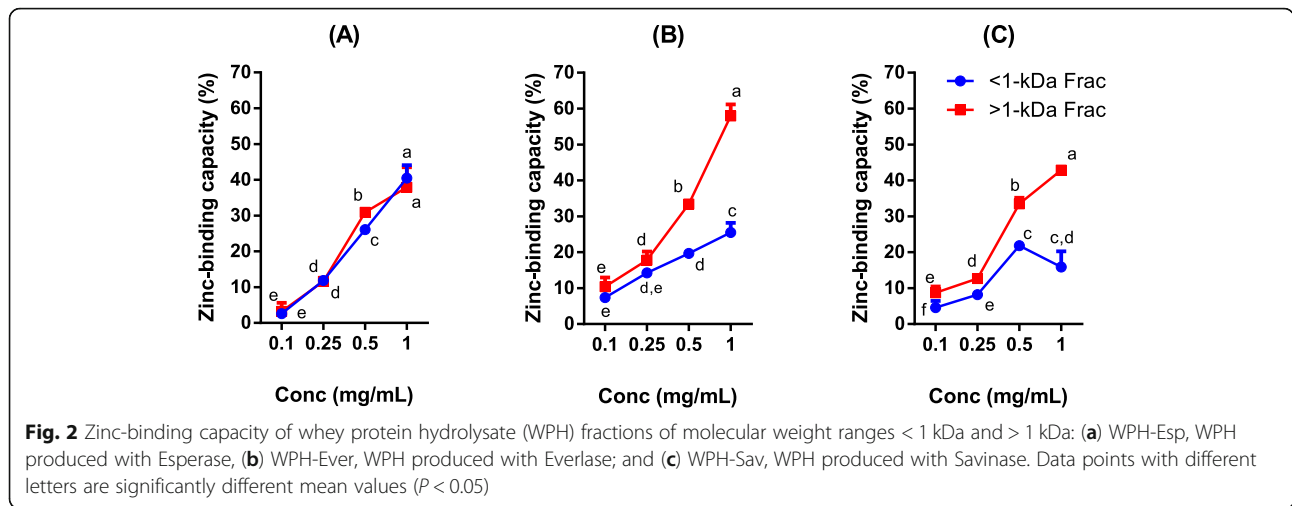
Compared to the hydrolysate and ultrafiltration fraction, the potent zinc-binding peptides led to a significantly ( $P < 0.001$ ) higher inhibition of enzymatic activity of ADAM17 (Fig. 3c). The IMAC-ZBP (1 mg/mL) was also more active (83% ADAM17 inhibition) than two rye secalin-derived tripeptides (CQV and QCA at 5  $\mu$ M) and their analogues (QCV and QVC at 5  $\mu$ M), which had  $\sim 70\%$  inhibition (Udechukwu et al. 2017). This demonstrates the feasibility of producing strong zinc ligands from food proteins for inhibiting zinc-dependent enzymes via zinc cofactor coordination. Synthetic compounds possessing ligands with higher affinity for zinc presented better ADAM17 inhibition than those with lower zinc-binding affinity (Shep- peck et al. 2007). Studies on food-derived zinc-binding peptides have focused on their application as dietary zinc carriers for enhancing zinc bioavailability (Udechukwu et al. 2016; Sun et al. 2020). This study is the first to demonstrate the systematic production of zinc-binding peptides from food proteins resulting in significantly enhanced ADAM17 inhibitory activity.

#### Identified peptides in IMAC-Zn<sup>2+</sup> fraction possess strong zinc ligands

The zinc-binding peptides were identified to evaluate their structural features that contributed to zinc binding. Shotgun peptidomics of IMAC-ZBP led to the identification of 24 peptides with chain length of 5 to 19 residues, net charge (pH7) of  $-3.997$  (anionic) to  $+1.997$  (cationic), and hydrophobicity of  $-2.585$  (hydrophilic) to  $1.300$  (hydrophobic). The sequence and physicochemical properties of the identified peptides are shown in Table 1. About 54% of the peptides corresponded with protein fragments of bovine  $\beta$ -lactoglobulin (10),  $\alpha$ -lactalbumin (1), and serum albumin (1), which are whey protein components. The remaining peptides were fragments of other bovine milk proteins viz.  $\beta$ -casein (9),  $\kappa$ -casein (1),

osteopontin-k (1), and folate receptor- $\alpha$  (1). The non-whey protein precursors were likely co-isolated with the whey proteins during processing. Four of the identified peptides contained oxidized Met residues, which may have occurred during protein isolation, hydrolysis, or storage (Yao and Udenigwe 2018). Aliphatic index of the peptides (ranged from 0 to 150) indicate a wide range of heat stability, with DTDY KKY and KIPAVF showing the lowest and highest stability, respectively. Notably, these two peptides were respectively the least and most hydrophobic as well (Table 1). Aliphatic index estimates the proportional heat stability of peptides based on the relative volume occupied by aliphatic side chains of proteins (Ikai 1980). However, only nine of the identified peptides, which have low-molecular weights, were stable based on the instability index. This estimates the stability of proteins in a test tube, with values above 40 indicating that the protein or peptide may be unstable (Guruprasad et al. 1990).

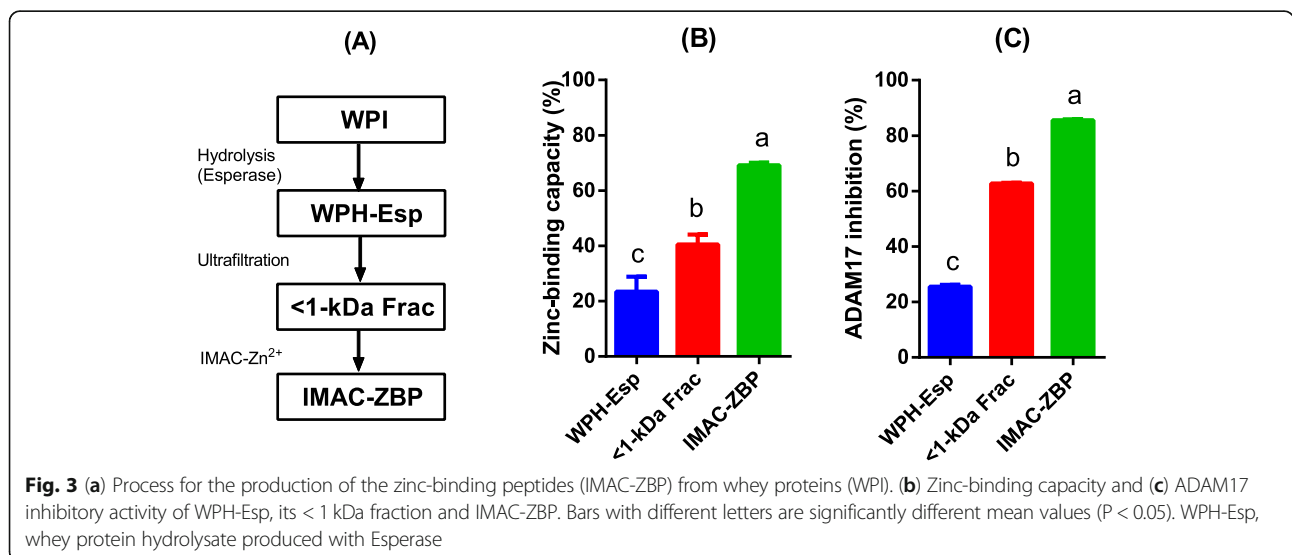
The structural features of the identified peptides clearly explain their high zinc-binding capacity. As shown in Table 1, the peptide sequences contain one or more of strong metal ligand groups His+Cys (H + C), Asp+Glu (D + E) and Try+Phe (Y + F); however, none of the peptides possess a Cys residue. High content of these amino acid residues is associated with the metal-binding capacity of peptides (Storcksdieck genannt Bonsmann, and Hurrell 2007; Torres-Fuentes et al. 2011; Carrasco-Castilla et al. 2012; Sun et al. 2020). His+Cys are well known structural features of zinc finger proteins where they function in zinc coordination; D + E and Y + F, on the other hand, bind divalent metals through electrostatic interaction and ring stacking/cation- $\pi$  interaction, respectively (Sun et al. 2020). Notably,  $\beta$ -lactoglobulin-derived cationic TMKGLIQ was identified in the fraction (Table 1), even though it lacks the strong



metal ligands. It is likely that the other residues, e.g. Thr (T), Met (M) and Gln (Q), contributed in binding of the heptapeptide to zinc during IMAC. The presence of N-terminal His residues in HHKAEPGPEDSLHEQ, HQPH QPLPPTVMFPPQ, and HKEMPFKYPVEPF may have also enhanced zinc binding. His residue at this position was reported to potentiate the metal-binding capacity of peptides (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara 1998; Zhu et al. 2015). Moreover, these peptides, especially HHKAEPGPEDSLHEQ, share some structural similarities (peptide length and His composition) with the ADAM17 catalytic site zinc structural motif (HEXXHXXGXXHD). Thus, the whey-derived peptide may have similar affinity for the zinc cofactor, leading to ADAM17 inhibition.

**Identified peptides were non-toxic but possessed poor pharmacokinetic properties based on in silico ADME/Tox analysis**

The ADME/Tox profile of pharmacologically active compounds provides an estimate of their safety and pharmacokinetic properties prior to in vivo studies and human consumption. In this study, in silico prediction indicated that the 24 peptides from the zinc-binding fraction (IMAC-ZBP) were non-toxins (Table 2); this demonstrates that they are potentially safe for human consumption. Estimated solubility (ESOL) evaluation indicates that the peptides ranged from highly soluble to soluble, which is prerequisite for ensuring that the peptide-zinc complex are bioaccessible in aqueous biological environments. However, evaluation of pharmacokinetics and drug-likeness using the validated SwissADME tool indicated that the 24 zinc-binding peptides have low passive gastrointestinal absorption (GIA).



**Table 1** Sequence, physicochemical properties and metal ligand content of zinc-binding peptides derived from whey protein hydrolysate

Peptide sequence	Parent protein	Accession #	Net charge, pH 7	Hydrophobicity	pI	Instability index < 40	Aliphatic index	% Total metal binding sites		
								%(H + C)	%(D + E)	%(Y + F)
HHKAPGEDSLHEQ	Folate receptor- $\alpha$	P02702	-1.725	-1.878	6.21	93.64	35	21.4	21.4	0
TPVWPPFLQPEVM	$\beta$ -casein	P02666	-1.000	0.800	3.84	113.77	110.71	0	7.14	7.14
VEELKPTPEGDLLEIL	$\beta$ -lactoglobulin	P02754	-3.995	-0.671	3.67	57.65	123.33	0	33.3	0
LVRTPEVDDE	$\beta$ -lactoglobulin	P02754	-2.997	-0.860	3.73	11.35	97	0	40.0	0
ELKPTPEGDLLEIL	$\beta$ -lactoglobulin	P02754	-2.996	-0.484	3.77	39.9	120	0	30.8	0
DHKSEEDKHLKIR	Osteopontin-k	P31098	0.183	-2.238	7.71	62.56	60	15.4	30.8	0
EKTKIPAVF	$\beta$ -lactoglobulin	P02754	0.999	-0.033	9.53	17.66	86.67	0	12.5	12.5
TPVWPPPLQPE	$\beta$ -casein	P02666	-1.000	0.209	3.84	124.56	114.55	0	9.10	0
KILDKVGIN	$\alpha$ -lactalbumin	P00711	0.997	0.200	9.53	-16.88	162.22	0	11.1	0
VIESPPEIN	K-casein	P02668	-1.998	-0.144	3.61	190.04	118.89	0	22.2	0
YWLAHK	$\alpha$ -lactalbumin	P00711	1.087	-0.616	9.30	36.93	81.67	16.7	0	16.7
KTKIPAVF	$\beta$ -lactoglobulin	P02754	1.997	0.400	10.80	18.61	97.5	0	0	12.5
SWMHQPHQPLPPTVM	$\beta$ -casein	P02666	0.179	-0.693	7.71	114.67	45.33	13.3	0	0
DTDYKKY	$\beta$ -lactoglobulin	P02754	-0.003	-2.585	6.43	-12.9	0	0	28.6	28.6
VRTPEVDDE	$\beta$ -lactoglobulin	P02754	-2.997	-1.377	3.73	11.5	64.44	0	44.4	0
WMHQPHQPLPPTVM	$\beta$ -casein	P02666	0.179	-0.685	7.71	122.14	48.57	14.3	0	0
TMKGLIQ	$\beta$ -lactoglobulin	P02754	0.997	0.242	9.70	-3.56	111.43	0	0	0
HQPHQPLPPTVMFPQ	$\beta$ -casein	P02666	0.179	-0.906	7.71	93.64	42.5	12.5	0	6.25
YWLAH	$\alpha$ -lactalbumin	P00711	0.088	0.040	7.54	-5.04	98	20.0	0	20.0
HKEMFPKYPVEPF	$\beta$ -casein	P02666	0.091	-1.000	7.54	135.19	20.71	7.14	14.3	21.4
LSFNPY	$\beta$ -lactoglobulin	P02754	-0.003	-0.100	6.09	3.53	65	0	0	33.3
FYAPPELL	Serum albumin	P02769	-1.001	0.828	3.85	141.31	125.71	0	14.3	28.6
KIPAVF	$\beta$ -lactoglobulin	P02754	0.997	1.300	9.70	21.48	130	0	0	16.7
SVMHQPHQPLPPTVMFPPQ	$\beta$ -casein	P02666	0.179	-0.480	7.71	110.58	51.05	10.5	0	5.26

Instability index, estimation of stability of protein in a test tube: a protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable (Guruprasad et al. 1990); Aliphatic index, positive factor for thermostability of globular proteins based on the relative volume occupied by aliphatic side chains of protein (Ikai 1980) (ExPASy ProtParam); % Total (potential) metal binding sites, manual calculations of (number of applicable amino acid/total chain length)\*100, %(H + C), %(D + E) and %(Y + F) (Lu et al. 2012) each represented different metal binding mechanisms

None of the peptides was predicted to inhibit cytochrome P450 3A4 (involved in drug metabolism), and all but three of the peptides are P-glycoprotein substrates (Table 2). Thus, the peptides could be rapidly removed from cells and metabolized in the body. The rotatable bonds (ROTB) and topological polar surface area (TPSA) of the 24 peptides were higher than 10 and 140 Å<sup>2</sup>, respectively (Table 2), which indicate that the zinc ligands have poor oral bioavailability. This result was expected as the peptides possess relatively long chains when compared to orally bioavailable food peptides, which typically have 2–4 amino acid residues.

## Conclusion

Food-derived peptides possess structural features that enable them to interact with and inactivate molecular disease targets. Findings from this study indicate that post-hydrolysis processing of whey peptides enhanced their biological activities. Particularly, zinc-binding peptides derived from WPH-Esp using IMAC possessed higher zinc-binding capacity compared to the parent protein hydrolysate and < 1-kDa peptide fraction. Peptides identified from the IMAC-ZBP had strong zinc amino acid ligands in their sequence, which facilitated their affinity for zinc and separation during IMAC. This enrichment resulted in a relatively higher inhibitory

**Table 2** In silico absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) profile of whey protein-derived zinc-binding peptides

Peptide sequence	Physiochemical properties					Toxicity SVM score (< 0.0)	Lipophilicity		Drug-likeness		Pharmacokinetics		
	MW (g/mol) < 500	ROTB (n) < 10	HBA (n) < 10	HBD (n) < 5	ESOL Log S		TPSA (Å <sup>2</sup> ) < 140	CLogP (o/w) < 5	Bioavailability score	Lipinski filter	GIA	P-glycoprotein substrate	CYP3A4 Inhibitor
HHKAPGPEDSLH EQ	1581.64	62	28	22	1.96 (HS)	-1.55 Non-toxin	711.32	-6.21	0.17	No	Low	Yes	No
TPVWPPFLQPE VM	1552.87	54	20	14	-5.97 (MS)	-1.16 Non-toxin	532.38	0.17	0.11	No	Low	Yes	No
VEELKPTPEGDLEIL	1681.88	69	29	21	-1.85 (VS)	-1.15 Non-toxin	685.59	-2.46	0.11	No	Low	Yes	No
LVRTPEVDDE	1172.24	47	22	18	-0.27 (VS)	-0.84 Non-toxin	547.76	-3.89	0.11	No	Low	Yes	No
ELKPTPEGDLEIL	1453.63	59	25	18	-1.13 (VS)	-1.41 Non-toxin	590.39	-2.16	0.11	No	Low	Yes	No
DHKSEEDKHLKIR	1634.79	73	30	27	4.89 (HS)	-0.84 Non-toxin	779.27	-6.05	0.17	No	Low	Yes	No
EKTIPAVF	1032.23	41	16	13	-0.61 (VS)	-1.45 Non-toxin	396.9	-1.06	0.17	No	Low	Yes	No
TPVWPPFLQPE	1175.37	39	17	11	-3.05 (S)	-1.25 Non-toxin	419.78	-1.14	0.11	No	Low	Yes	No
KILDKVGIN	999.21	44	16	14	0.42 (HS)	-1.27 Non-toxin	428.55	-1.52	0.17	No	Low	Yes	No
VIESPPEIN	997.1	37	17	12	-0.81 (VS)	-0.43 Non-toxin	416.46	-2.18	0.11	No	Low	Yes	No
YWLAHK	816.95	28	11	11	-2.51 (S)	-1.14 Non-toxin	299.54	0.89	0.17	No	Low	Yes	No
KTKIPAVF	903.12	35	13	11	-2.14 (S)	-1.37 Non-toxin	330.52	0.18	0.17	No	Low	Yes	No
SWMHQPHQPLPPTVM	1786.08	62	23	19	-4.09 (MS)	-1.29 Non-toxin	685.95	-2.33	0.17	No	Low	Yes	No
DTDYKKY	931.99	36	18	15	2.57 (HS)	-0.51 Non-toxin	425.25	-3.33	0.17	No	Low	Yes	No
VRTPEVDDE	1059.08	42	21	17	0.77 (HS)	-0.79 Non-toxin	518.66	-4.25	0.11	No	Low	Yes	No
WMHQPHQPLPPTVM	1699.01	58	21	17	-4.64 (MS)	-1.22 Non-toxin	636.62	-1.4	0.17	No	Low	Yes	No
TMKGLIQ	789.98	34	12	11	-0.19 (VS)	-0.85 Non-toxin	352.56	-1.04	0.17	No	Low	No	No
HQPHQPLPPTVM FPPQ	1851.14	62	24	17	-4.15 (MS)	-1.03 Non-toxin	679.24	-3.37	0.17	No	Low	Yes	No



**Table 2** In silico absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) profile of whey protein-derived zinc-binding peptides (Continued)

Peptide sequence	Physicochemical properties					Toxicity SVM score (< 0.0)	Lipophilicity		Drug-likeness		Pharmacokinetics		
	MW (g/mol) < 500	ROTB (n) < 10	HBA (n) < 10	HBD (n) < 5	ESOL Log S		TPSA (Å <sup>2</sup> ) < 140	CLogP (o/w) < 5	Bioavailability score	Lipinski filter	GIA	P-glycoprotein substrate	CYP3A4 Inhibitor
YWLAH	688.77	21	9	9	-2.52 (S)	-0.88 Non-toxin	244.42	0.69	0.17	No	Low	No	No
HKEMFPKYPVE PF	1746.04	62	24	17	-3.34 (S)	-1.32 Non-toxin	607.31	-1.09	0.17	No	Low	Yes	No
LSFNPY	739.82	24	11	9	-1.50 (VS)	-0.85 Non-toxin	283.58	-0.4	0.17	No	Low	Yes	No
FYAPPELL	851.98	29	12	9	-2.87 (S)	-1.42 Non-toxin	286.66	0.95	0.11	No	Low	Yes	No
KIPAVF	673.84	24	9	7	-2.32 (S)	-1.1 Non-toxin	226.05	0.75	0.17	No	Low	No	No
SVMHQPHQLPP TVMFPPQ	2168.54	76	28	21	-5.05 (MS)	-0.76 Non-toxin	812.07	-4.34	0.17	No	Low	Yes	No

Abbreviations: MW molecular weight (g/mol); ROTB (n) rotatable bonds; HBA (n) hydrogen bond acceptors; HBD (n) hydrogen bond donors; ESOL estimated solubility based on (Delaney 2004) with solubility classes in bracket (HS highly soluble, VS very soluble, MS moderately soluble, S soluble); Toxicity SVM score (BIOPEP and ToxinPred), support vector machine score based on (Gupta et al. 2013); TPSA (Å<sup>2</sup>) topological polar surface area; CLogP (o/w) logarithm of compound partition coefficient between n-octanol and water; Bioavailability score, probability of F > 10% in rat, implemented from (Martin 2005); Lipinski filter (based on Lipinski rules of 5, all peptides showed 3 violations); GIA, gastrointestinal absorption; P-glycoprotein substrate, permeability-glycoprotein substrate SVM model (SwissADME); CYP3A4, cytochrome P450 3A4

effect of the IMAC-ZBP against the enzymatic activity of zinc-dependent ADAM17. The peptides were predicted to be non-toxins but to have poor oral bioavailability. Thus, the zinc-binding peptides may be further explored for use in cases where intestinal absorption and cellular uptake are not required, e.g. in controlling aberrant TNF- $\alpha$ -mediated inflammatory processes in the gut. Lastly, this study provided several food-derived zinc-binding peptide sequences for future structure-function relationship studies and the development of functional foods and nutraceuticals against inflammatory and other zinc-related diseases.

#### Abbreviations

IMAC: Immobilized metal affinity chromatography; WPI: Bovine whey protein isolate; SDS: Sodium dodecyl sulphate; DTT: Dithiothreitol; CV: Column volume; TFA: Trifluoroacetic acid; LC-MS/MS: Liquid chromatography-mass spectrometry; ROTB: Rotatable bonds; TPSA: Topological polar surface area

#### Acknowledgements

Not applicable.

#### Authors' contributions

MCU designed experiments, collected and analyzed data, and co-prepared the first draft of the manuscript. CD collected and analyzed data. CCU designed experiments, collected and analyzed data, co-prepared the first draft, read and edited the manuscript. All authors read and approved the final manuscript.

#### Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants (RGPIN-435865-2013 and RGPIN-

2018-06839), and Canada Foundation for Innovation (CFI) John R. Evans Leaders Fund Infrastructure Grant (Project number: 31305).

#### Availability of data and materials

All the data supporting the results are included within the article.

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

Authors declare that they have no competing interests.

#### Author details

<sup>1</sup>Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia B2N 5E3, Canada.

<sup>2</sup>Department of Chemistry and Biomolecular Sciences, Faculty of Science, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada. <sup>3</sup>School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada.

Received: 7 November 2020 Accepted: 15 December 2020

Published online: 02 February 2021

#### References

- Agyei, D., Tsopmo, A., & Udenigwe, C. C. (2018). Bioinformatics and peptidomics approaches to the discovery and analysis of food-derived bioactive peptides. *Analytical and Bioanalytical Chemistry*, 410(15), 3463–3472.
- Bischoff, R., & Schlüter, H. (2012). Amino acids: Chemistry, functionality and selected non-enzymatic post-translational modifications. *Journal of Proteomics*, 75(8), 2275–2296.

- Block, H., Maertens, B., Spriestersbach, A., Brinker, N., Kubicek, J., Fabis, R., & Schäfer, F. (2009). Chapter 27 immobilized-metal affinity chromatography (IMAC). A review. *Methods in Enzymology*, 463, 439–473.
- Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Giron-Calle, J., & Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. *Food Chemistry*, 135(3), 1789–1795.
- Chen, D., Liu, Z., Huang, W., Zhao, Y., Dong, S., & Zeng, M. (2013). Purification and characterisation of a zinc-binding peptide from oyster protein hydrolysate. *Journal of Functional Foods*, 5(2), 689–697.
- Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of Histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49–53.
- Cheung, R. C. F., Wong, J. H., & Ng, T. B. (2012). Immobilized metal ion affinity chromatography: A review on its applications. *Applied Microbiology and Biotechnology*, 96, 1411–1420.
- Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, 7, 42717. <https://doi.org/10.1038/srep42717>.
- Delaney, J. S. (2004). ESOL: Estimating aqueous solubility directly from molecular structure. *Journal of Chemical Information and Computer Sciences*, 44, 1000–1005.
- Gaberc-Porekar, V., & Menart, V. (2001). Perspectives of immobilized-metal affinity chromatography. *Journal of Biochemical and Biophysical Methods*, 49(1–3), 335–360.
- Gardner, M. L. (1988). Gastrointestinal absorption of intact proteins. *Annual Review of Nutrition*, 8, 329–350.
- Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Consortium, O. S. D. D., & Raghava, G. P. S. (2013). *In silico* approach for predicting toxicity of peptides and proteins. *PLoS One*, 8(9), e73957. <https://doi.org/10.1371/journal.pone.0073957>.
- Guruprasad, K., Reddy, B. V. B., & Pandit, M. W. (1990). Correlation between stability of a protein and its dipeptide composition: A novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Engineering*, 4, 155–161. <https://doi.org/10.1093/protein/4.2.155>.
- Ikai, A. J. (1980). Thermostability and aliphatic index of globular proteins. *Journal of Biochemistry*, 88, 1895–1898.
- Jakob, U., Eser, M., & Bardwell, J. C. A. (2000). Redox switch of Hsp33 has a novel zinc-binding motif. *Journal of Biological Chemistry*, 275(49), 38302–38310.
- Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16(9), 945–960.
- Krissansen, G. W. (2007). Emerging health properties of whey proteins and their clinical implications. *Journal of the American College of Nutrition*, 26(6), 713–723.
- Lu, C., Lin, Y., Lin, J., & Yu, C. (2012). Prediction of metal ion-binding sites in proteins using the fragment transformation method. *PLoS One*, 7(6), e39252. <https://doi.org/10.1371/journal.pone.0039252>.
- Martin, Y. C. (2005). A bioavailability score. *Journal of Medicinal Chemistry*, 48(9), 3164–3170. <https://doi.org/10.1021/jm0492002>.
- Rajendran, S. R. C. K., Mason, B., & Udenigwe, C. C. (2016). Peptidomics of peptic digest of selected potato tuber proteins: Post-translational modifications and limited cleavage specificity. *Journal of Agricultural and Food Chemistry*, 64, 2432–2437. <https://doi.org/10.1021/acs.jafc.6b00418>.
- Remko, M., Fitz, D., Broer, R., & Rode, B. M. (2011). Effect of metal ions (Ni<sup>2+</sup>, Cu<sup>2+</sup> and Zn<sup>2+</sup>) and water coordination on the structure of L-phenylalanine, L-tyrosine, L-tryptophan and their zwitterionic forms. *Journal of Molecular Modeling*, 17(12), 3117–3128. <https://doi.org/10.1007/s00894-011-1000-0>.
- Shepck, J. E., Tebben, A., Gilmore, J. L., Yang, A., Wasserman, Z. R., Decicco, C. P., & Duan, J. J. W. (2007). A molecular modeling analysis of novel non-hydroxamate inhibitors of TACE. *Bioorganic and Medicinal Chemistry Letters*, 17(5), 1408–1412.
- Storcksdieck Genannt Bonsmann, S., & Hurrell, R. F. (2007). Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *Journal of Food Science*, 72(1), S019–S029.
- Sun, X., Sarteshnizi, R. A., Boachie, R. T., Okagu, O. D., Abioye, R. O., Pfeilsticker Neves, R., ... Udenigwe, C. C. (2020). Peptide–mineral complexes: Understanding their chemical interactions, bioavailability, and potential application in mitigating micronutrient deficiency. *Foods*, 9(1402), 1–17.
- Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic*, 90, 1–11.
- Torres-Fuentes, C., Alaiz, M., & Vioque, J. (2011). Affinity purification and characterisation of chelating peptides from chickpea protein hydrolysates. *Food Chemistry*, 129(2), 485–490.
- Udechukwu, M. C., Collins, S. A., & Udenigwe, C. C. (2016). Prospects of enhancing dietary zinc bioavailability with food-derived zinc-chelating peptides. *Food Function*, 7(10), 4137–4144.
- Udechukwu, M. C., Downey, B., & Udenigwe, C. C. (2018). Influence of structural and surface properties of whey-derived peptides on zinc-chelating capacity, and in vitro gastric stability and bioaccessibility of the zinc-peptide complexes. *Food Chemistry*, 240, 1227–1232.
- Udechukwu, M. C., Tsopmo, A., Mawhinney, H., He, R., Kienesberger, P. C., & Udenigwe, C. C. (2017). Inhibition of ADAM17/TACE activity by zinc-chelating rye secalin-derived tripeptides and analogues. *RSC Advances*, 7, 26361–26369.
- Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing, and potential health benefits. *Journal of Food Science*, 77, R11–R25.
- Walters, M. E., Esfandi, R., & Tsopmo, A. (2018). Potential of food hydrolyzed proteins and peptides to chelate iron or calcium and enhance their absorption. *Foods*, 7(10), 172.
- Walzem, R. L., Dillard, C. J., & German, J. B. (2002). Whey components: Millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking. *Critical Reviews in Food Science and Nutrition*, 42(4), 353–375.
- Wang, C., Li, B., & Ao, J. (2012). Separation and identification of zinc-chelating peptides from sesame protein hydrolysate using IMAC-Zn<sup>2+</sup> and LC-MS/MS. *Food Chemistry*, 134(2), 1231–1238.
- Xie, N., Huang, J., Li, B., Cheng, J., Wang, Z., Yin, J., & Yan, X. (2015). Affinity purification and characterisation of zinc chelating peptides from rapeseed protein hydrolysates: Possible contribution of characteristic amino acid residues. *Food Chemistry*, 173, 210–217.
- Yao, S., & Udenigwe, C. C. (2018). Peptidomics analysis of potato protein hydrolysates: Implications of post-translational modifications in food peptide structure and behaviour. *Royal Society Open Science*, 5, 172425. <https://doi.org/10.1098/rsos.172425>.
- Zhang, J., Xin, L., Shan, B., Chen, W., Xie, M., Yuen, D., ... Ma, B. (2012). PEAKS DB: *de novo* sequencing assisted database search for sensitive and accurate peptide identification. *Molecular and Cellular Proteomics*, 11(4), M111.010587.
- Zhu, K. X., Wang, X. P., & Guo, X. N. (2015). Isolation and characterization of zinc-chelating peptides from wheat germ protein hydrolysates. *Journal of Functional Foods*, 12, 23–32.

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://biomedcentral.com/submissions)

