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Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates

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ABSTRACT

Buffalo and bovine caseins were hydrolysed by alcalase and trypsin to produce novel antioxidant peptides. The casein hydrolysates were purified using ultrafiltration (UF) and further characterized by RP-HPLC. The fractions produced higher antioxidant activities were identified for their peptides using LC MS/MS. All UF-VI (MW < 1 kDa) fractions showed higher antioxidant activity. Hydrolysate produced by alcalase for buffalo casein (UF-VI with 54.84-fold purification) showed higher antioxidant activity than that obtained by trypsin. Trypsin hydrolysate contained high amount of hydrophobic amino acids while alcalase hydrolysate consisted mainly of Ser, Arg, Ala and Leu. The antioxidant peptides identified by LC MS/MS were RELEE, MEDNKQ and TVA, EQL in buffalo casein hydrolysates produced by trypsin and alcalase, respectively. Mechanism and reaction pathways of selected antioxidant peptides with ABTS were proposed. Conclusively, buffalo casein provided antioxidant peptides similar to bovine, suggesting that buffalo casein is a novel source of antioxidant.

1. Introduction

Bioactive peptides derived from milk proteins are short protein sequences, which are inactive within the parent protein but may reveal some biofunctional and physiological activities after releasing from their protein sources (Behera et al., 2013). Depending on their amino acid contents, sequences and structures, peptides generated can play different functions, such as immunomodulatory, antimicrobial, antioxidative, *anti*-thrombotic hypocholesterolemic and angiotensin converting enzyme (ACE) inhibitory (Meisel, 2005). Cow milk proteins are considered excellent and the primary source of specified bioactive peptides due to their diverse physiological activity. However, the main buffalo milk proteins show high homology as compared to the cow milk proteins, in particular some amino acid sequences and structures (D'Ambrosio et al., 2008). Buffalo milk is ranked at second after cow milk, based on worldwide milk production and distribution with leading countries in Asia, the Middle East and Europe. Notably, about 103 million tons of buffalo milk was produced in 2013, representing 13% of the total world milk production with an annual growth rate of ~3.3% which is higher than cow milk (annual growth rate 0.9%) (IDF, 2014). Buffalo milk proteins are potential precursors for bioactive peptides with versatile functionalities including antioxidant activity (Clare & Swaisgood, 2000; Power, Jakeman, & FitzGerald, 2013), antibacterial, ACE inhibitory activities and supporting the growth of bifidobacteria (D'Ambrosio et al., 2008). Antioxidant peptides derived from milk proteins are important compounds attributing for extending the shelf life of processed foods (Hogan, Zhang, Li, Wang, & Zhou, 2009). In addition, they prevent oxidative stress in plasma from excessive free radical production consequently reduce the risk of aging (Power-Grant et al., 2016). Many studies have been carried out on isolation and identification of bioactive peptides from cow milk proteins including both casein and whey (De Gobba, Espejo-Carpio, Skibsted, & Otte,

Abbreviations: CB, buffalo casein; CN, bovine casein; CBH, buffalo casein hydrolysate; CNH, bovine casein hydrolysate; UF, ultrafiltration; ABTS, 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); RP-HPLC, reversed-phase high performance liquid chromatography; MW, molecular weight; LC–MS, liquid chromatography mass spectrometry; LC–MS/MS, Liquid Chromatography–Tandem Mass Spectrometry.

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2014; Hu et al., 2016). However, studies on buffalo milk proteins were limited to the buffalo whey proteins only and less work was reported as regarding to buffalo casein. Although, Shanmugam, Kapila, Sonfack, and Kapila (2015) studied the antioxidant peptides from buffalo casein but still more work is needed to further identify some new sequence of amino acids with higher bioactivity. Antioxidant peptides can be produced from fermentation and by using commercial enzymes (Power et al., 2013). Many studies have reported the ABTS assay is preferable in confirming the antioxidant activity of peptides for different foods, due to their more sensitive, given the stability and ease to generate the radical form of ABTS than other method (Becker, Nissen, & Skibsted, 2004; Zhu, Jie, Tang, & Xiong, 2008). Additionally, their donating free radicals give similar findings in the biological system (oxidize nucleic acids, proteins, lipids, or DNA) (Cheng, Xiong, & Chen, 2010; Jaiswal, Bajaj, Mann, & Lata, 2015).

There is little information about the antioxidant activity of buffalo milk proteins, their hydrolysates and the structure of antioxidant peptides. Therefore, in the present study, we compared the antioxidant activity of buffalo and bovine casein hydrolysates (CBH and CNH) through ABTS assay, that were prepared by enzymatic hydrolysis using both alcalase and trypsin. UF and preparative high performance liquid chromatography were used for fractionation and purification of the antioxidant peptides. In addition, the antioxidant peptides from hydrolysates with highest antioxidant activity were identified by tandem mass spectrometry (MS/MS).

2. Materials and methods

2.1. Materials

Sodium caseinate from bovine milk (CN) was purchased from Tokyo chemical industry Co., Ltd. (Tokyo, Japan). The 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2, 5,7,8-tetramethylchroman-2-car-boxylic acid (Trolox) were purchased from Aldrich Chemical Co., Ltd. (Shanghai, China). Trypsin (EC no 3.4.21.4, pH range 7–9, and 37 °C) and alcalase (EC no of 3.4.21.62, pH range 5–7 and 55 °C) were purchased from Novo Enzyme (Bagsvaerd, Denmark). All other chemicals used in the experiments were of analytical grade.

2.2. Preparation of sodium caseinate from buffalo milk (CB)

Skim milk was prepared from fresh whole buffalo milk obtained from the Farm of Faculty of Agriculture (Cairo University, Cairo, Egypt) by centrifugation (Milk centrifuge Elecrem, Helmut Rink GmbH, Amtzell, Germany) at 2000×*g* for 30 min at 4 °C. The average composition of skim milk in dry weight was 10.62, 0.1, 4.23, 4.92, and 0.97% for total solids, fat, protein, lactose and ash, respectively.

Sodium caseinate from buffalo milk was prepared according to the method of Mulvihill (1992) with some modifications. Skim milk was acidified to pH 4.6 with hydrochloric acid (1 M) under continuous stirring by stirrer (IKA@-works guangzhou Co., Guangzhou, China) at 25 °C. After leaving of curd deposition for 20 min, the mixture was filtered (Whatman No. 40). The precipitated casein was washed with distilled water, then dissolved with the addition of NaOH (1 M) at pH 7.0, and again left for precipitation. Precipitation and washing steps were repeated four times. The final precipitates were dissolved in NaOH (1 M) to pH 7.0, thereafter heated at 80 °C for 30 min to inactivate plasmin, dialysed against distilled water and lyophilized.

2.3. Preparation of casein hydrolysates

Casein solution (2% w/w on protein basis) was prepared by dissolving ~2.2 g of casein powder (according to percent of protein in casein powder) in 100 g distilled water; dispersion was stirred for 1 h at room temperature and kept overnight at 5 °C. On the next day, 80 mg/mL enzyme solution (dissolved in distilled water) was added to make the hydrolysates. The pH and temperature of the casein solution was adjusted according to the characteristics of the enzymes as described above. The enzymes were pre-incubated for 10 min and the enzyme to substrate ratio 1:100 (w/w) was used in all experiments. The hydrolysis process was started by adding the enzyme solution to the casein solution with 30 s vortexes. The reaction mixture was quenched after 3 h of hydrolysis, then heated at 90 °C for 15 min to stop the enzyme activity followed by cooling (20 min in ice bath) and centrifugation (Model 3K16, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) (10 min at 12,000×*g*). The supernatant was used for further analyses such as total amino acids composition and ABTS assay, as described by Otte, Shalaby, Zakora, Pripp, and El-Shabrawy (2007).

2.4. Preparation of peptide fractions

The hydrolyzed solution was separated by centrifugation (Model 3K16, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 12,000×*g* for 10 min at 4 °C and filtered through Whatman No. 40 filter (0.45 μm, 90 mm filter holder, CAT NO. YY 3009000, EMD Millipore Co., Billerica, MA, USA) and fractioned to different stages by using ultrafiltration (UF) membranes with molecular mass cut off sizes (30, 10, 5, 3 and 1 kDa) (Millipore, Barnant Co., Assembled, USA). Permeate and retentate of each stage of filtration were collected and stored at −20 °C until further experiments. Protein concentrations in all fractionated protein solutions were determined by the biuret method.

2.5. Determination of total amino acids composition

Amino acid composition of both casein and the hydrolysates was determined as described earlier by Adeyeye (2009) with slight modifications. Glass ampoules were used to insert 30 mg of casein sample or 4 mL hydrolysate and 7 mL of 6 M HCl. The glass ampoules were sealed with a flame and heated at 110 ± 5 °C for 22 h, followed by cooling and filtration. The filtrate was evaporated to dryness at 40 °C under vacuum. The residue was dissolved using 5 mL acetate buffer (pH 2.0). Each sample (1 μL) was injected into a ZORBAX 80A C_{18} column (4.6 × 180 mm, Agilent Technologies) at 40 °C with detection at 338 nm. Mobile phase A was 7.35 mM sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mM sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). Amino acids composition was expressed as g of amino acid per 100 g of protein. The amount of amino acids was calculated on the basis of the peak area in comparison with their reference standards.

2.6. Fractionation of the UF-VI by RP-HPLC

The UF-VI fraction obtained from UF was analyzed by RP-HPLC on a ZORBAX 300SB (Agilent Technologies In c., Palo Alto, CA, USA), X-Bridge OBD C_{18} column (30 × 250 mm, 5 μm), with a linear gradient of solvent B acetonitrile (0.1% TFA), solvent A (0.1% TFA in water) for 45 min at 25 °C with flow rate of 1 mL/min. Collection of fractions was made automatically (Fraction Collector, Agilent Technologies Series 1200) with a wavelength of absorbance monitored at 280 nm. The injection volume was 200 μL and the concen-

tration of protein material applied was approximately equivalent to 0.5 mg/mL. All samples were filtered through a 0.2 mm syringe filter prior application to the C_{18} column. Each of the major peaks was pooled from the 4 type UF-VI fractions and determined the peptide content by Lowry's method.

2.7. Estimation of ABTS radical scavenging activity

Radical scavenging activity was tested using the method of Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005) with some modification. ABTS radical cation ($ABTS^+$) was prepared 12–16 h before using into a 25 mL volumetric flask by dissolving 7 mM ABTS stock solution with 2.45 mM potassium persulfate in distilled water and the solution was kept at room temperature in the absence of light. The $ABTS^+$ solution was diluted in 5 mM phosphate buffer saline (PBS, pH 7.4), and used for setting absorption at 734 nm in a 3 cm cuvette until absorbance value of 0.70 ± 0.02 was achieved at 30 °C. Standard curve was obtained by adding 2 mL of diluted $ABTS^+$ solution to 20 μL of Trolox with a final concentration of Trolox ranging from 0 to 8 mg/mL in PBS. The absorbance reading was taken after 10 min at 30 °C. Appropriate blank solvent was run in each assay. Activity of each 20 μL sample was measured in triplicate. The percent of inhibition was calculated as a function of antioxidant concentration corresponding to Trolox as a standard compound. To calculate the Trolox Equivalent Antioxidant Capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance sample concentration was divided by the gradient of the plot for Trolox. This gave the TEAC at a specific time.

2.8. Analysis by RP-HPLC-MS/MS

The fractions obtained by RP-HPLC with the highest antioxidant activity were characterized for peptides identification. The LC–MS/MS analysis was done according to Otte et al. (2007) with a slight modification. The LC-MS analysis was performed using an Acquity Ultra UPLC System (Waters, Milford, MA, USA) through an electrospray ionization source. The MS analysis was carried out by a tandem mass spectrometer composed of a quadruple combined with a time-of-flight (WATERS MALDI SYNAPT QTOF MS, Waters, Milford, MA, USA). UPLC was equipped with Acquity UPLC BEH C_{18} column (150 × 2.1 mm). 1 μL was injected and elected using a flow rate of 0.3 mL/min and gradient consisting of 0–60% acetonitrile (solvent B) in 40 min. Mobile phase A consisted of 0.1% formic acid in water. The fractions obtained by RP-HPLC were used to determine the amino acid sequences of the identified peptides. Peptide profiles were recorded from MS/MS spectra in the range of 50–2000 m/z (depending on the m/z and the charge state of the precursor ion) at the normal scan resolution and the target mass was set to 1521 m/z to the identification of amino acid sequence using a scan time of 0.5 s, capillary voltage of 2 kV, source temperature of 100 °C, and the desolation gas temperature of 400 °C. The data obtained was analyzed using the software of Mass Lynx V4.1, MassEnt3 and BioLyxn Peptide Sequencing.

2.9. Statistical analysis

All experiments were performed in triplicate. An analysis of variance (ANOVA) was performed, and the mean comparisons were analyzed by Duncan's multiple range test. The statistical analyses were accomplished using a SPSS package (SPSS 16.0 for Windows, SPSS Inc.).

3. Results and discussions

3.1. Fractionation of CBH and CNH by ultrafiltration

Ultrafiltration applications involve purification, division and making concentrate of several fractions of protein hydrolysates through the molecular-weight-cut off (MWCO) membrane. Therefore, basic system attributed to separate particles distributed in solution depending on molecular size (Hu, Chi, Wang, & Deng, 2015). In this study, CBH and CNH were fractionated into six different molecular weight (MW) fractions and named as UF-I (MW > 30 kDa), UF-II (10–30 kDa), UF-III (5–10 kDa), UF-IV (3–5 kDa), UF-V (1–3 kDa) and UF-VI (MW < 1 kDa). ABTS radical scavenging activity (percent of inhibition ± SD) and protein content of separated fractions are shown in Fig. 1. Generally, the inhibition percent of fraction UF-VI was higher than other fractions for alcalase and trypsin hydrolysate, which demonstrated that the antioxidant activity of UF-VI significantly increased after ultrafiltration separation. It was observed that fraction UF-VI with the lowest MW exhibited the highest antioxidant activities as compared to the other fractions. Similar results were reported by other

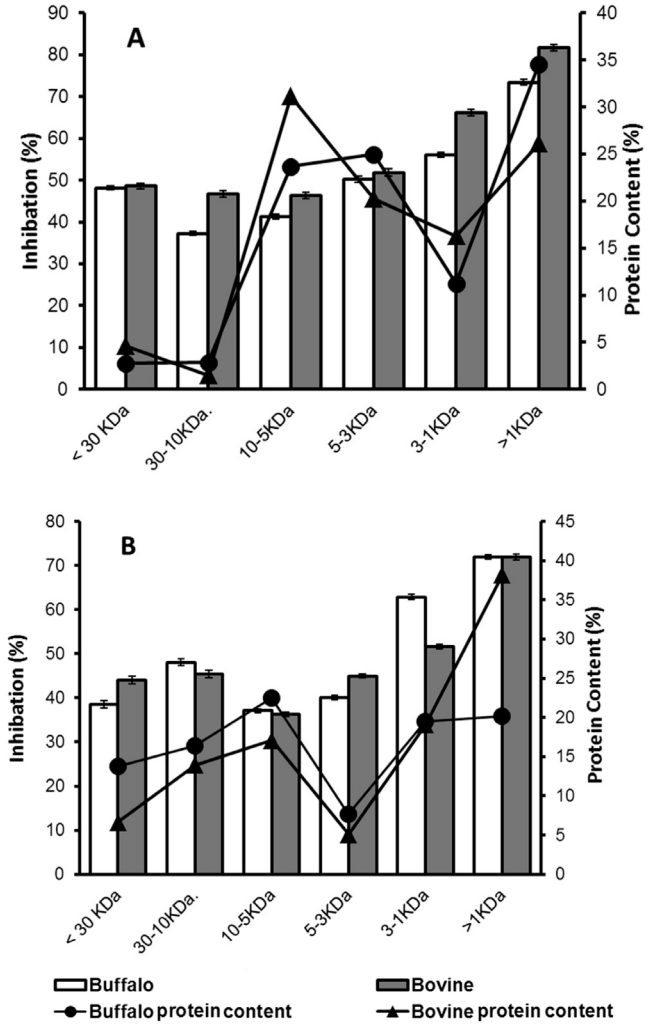

Fig. 1. Antioxidant activity as percent of inhibition (column) and protein content (line) of CNH and CBH fractions by (A) Alcalase (B) Trypsin after ultrafiltration. All data are presented as the mean ± SD of triplicate, different letters show significant differences ($p < 0.05$).

researchers for buffalo casein and sweet potato protein hydrolysates that the highest antioxidant activity was contributed by peptides containing high amount of low MW < 1500 Da (Shanmugam et al., 2015; Zhang, Huang, & Jiang, 2014). Alcalase hydrolysates exhibited significantly higher inhibition in UF-VI (73.44% for CBH and 81.69% for CNH) as compared to trypsin hydrolysates (71. 87% for CBH and 71. 95% for CNH) (see Table 1).

The protein content of CNH and CBH were different for all UF fractions. The fraction UF-VI, as expected had the highest protein contents of 38.16% for trypsin CNH and 34.54% for alcalase CBH. In contrast, protein contents in UF-III for alcalase CNH and trypsin CBH samples were 31.15 and 22.51%, respectively. Despite, UF-VI fractions of alcalase CNH and trypsin CBH displayed the highest antioxidant activity but, their protein contents (26.15 and 20.2%, respectively) were lower than other fractions. Analyses of the UF-processed hydrolysates demonstrated that the higher molecular mass substances were collected. Concentration using the UF technique resulted in an enrichment of the lower molecular mass peptides (MW < 1 kDa). This result confirmed that hydrolysis improved the radical scavenging activity. UF-VI composed of bioactive peptides lower than 1 kDa displayed the highest antioxidant activity. Moreover, the UF isolated fractions showed higher radical scavenging activity than the whole hydrolysate. An increase in the purification fold was observed at each step of purification as given in Table 2. Among all the UF fractions, UF-VI showed the highest purification fold. Furthermore, UF-VI alcalase CBH showed the highest value (54.84) as compared to other UF-VI fractions like alcalase CNH, trypsin CBH, and trypsin CNH (32.55, 36.65 and 40.23, respectively). These findings were in accordance with the results obtained by Irshad, Kanekanian, Peters, and Masud (2015) who reported an increase in antioxidant activity with the decrease in MW. After the fractionation process, small MW peptides could be obtained from casein hydrolysate, which showed more excellent antioxidant efficiency than the whole hydrolysates containing peptides with different MW. Zarei et al. (2014) explained that small-sized bioactive peptides with lower MW could cross the intestinal barrier intact and showed their antioxidant activity at the tissue. Therefore, the UF-VI (MW < 1 kDa) was selected for further study.

3.2. Amino acids composition of UF-VI fraction

The amino acid composition (involve in oxidative reactions) and the amount of hydrophobic amino acids (aromatic acids and basic amino acids) directly linked to the radical scavenging activities (Rajapakse, Mendis, Byun, & Kim, 2005). The amino acid compositions of CN, CB and its UF-VI (MW < 1 kDa) hydrolysates were shown in Table 3. Amino acids of UF-VI (MW < 1 kDa) had high free radical scavenging ability, due to the fact that amino acids had the possibility to donate protons readily to electron-negative radicals in oxidative system and kept their stability via resonance structures (Rajapakse et al., 2005). Therefore, the antioxidant activities of the UF-VI were attributed to the types of amino acids in the peptides. Amino acid composition of UF-VI showed the presence of more hydrophobic amino acids compared to CN and CB, which contained low molecular weight peptides. Although, there were many hydrophobic amino acids in UF-VI trypsin CBH compared to those in trypsin CNH, UF-VI trypsin CBH showed less inhibition activity than UF-VI trypsin CNH. Fraction UF-VI trypsin CBH contained the highest contents of some individual hydrophobic amino acids, such as threonine (3.38 mg), valine (6.8 mg), methionine (3.34 mg), isoleucine (4.91 mg), and glycine (22.07 mg). But, trypsin CNH contained the highest contents of total hydrophobic amino acids (61.7 mg), which could be the contributing factor for higher antioxidant activity. On the other hand, no difference in amino acid amounts was noted for alcalase hydrolysates of CN and CB (Jaiswal et al., 2015). Both CN and CB treated with alcalase showed the highest amount of amino acids including serine, arginine, alanine and leucine as compared to trypsin hydrolysis. These findings might be ascribed to a broad specificity of alcalase, which induced casein protein easily attacked on the peptide (amide) bond through a serine residue at the active site, thereby leading to large amounts of small peptides and free amino acids.

3.3. Fractionations of UF-VI trypsin CBH and CNH by semi-preparative RP-HPLC

In RP-HPLC, the separation was based on the hydrophilic and hydrophobic property, which meant non-polar or hydrophobic

Table 1
Amino acid composition (expressed as% of total amino acids) of CN, CB and UF-VI (MW < 1 kDa) fractions separated from casein hydrolysate by ultrafiltration.

Amino Acids	UF-VI (MW < 1 kDa)				CN[a]	CB[b]
	Trypsin		Alcalase			
	CNH	CBH	CNH	CBH		
Asp	6.16	6.23	5.65	5.70	6.74	5.26
Glu	21.23	22.07	21.51	21.51	22.21	18.91
Ser	3.70	3.74	4.15	4.01	4.29	3.74
His	2.77	2.47	2.57	2.60	2.89	2.19
Gly	1.94	1.89	1.86	1.86	1.74	1.46
Thr	3.32	3.38	3.32	3.30	3.60	3.51
Arg	2.70	2.57	3.50	3.50	3.64	2.48
Ala	3.18	3.03	3.38	3.38	2.90	2.33
Tyr	3.31	3.42	5.84	5.80	5.33	4.36
Cys-s	0.08	0.09	0.05	0.05	0.08	0.07
Val	6.75	6.80	6.53	6.56	6.66	5.64
Met	3.13	3.34	2.73	2.77	2.65	2.41
Phe	6.05	5.64	5.68	5.69	4.94	4.30
Ile	4.63	4.91	4.05	4.07	4.74	4.90
Leu	10.71	10.30	11.99	12.00	8.15	7.53
Lys	6.59	7.07	6.24	6.22	7.89	6.44
Pro	13.75	13.04	10.94	10.99	9.77	12.16
Hydrophobic group[a]	61.71	60.65	58.35	58.57	58.01	54.58
Ionzabled[b]	39.46	40.42	39.48	39.52	39.87	43.70

CN: Bovin Casein, CB: Buffalo Casein.
[a] Hydrophobic R groups include amino acids Ala, Val, Met, Phe, Ile, Leu, Pro, and Trp.
[b] Ionizable R groups include amino acids Lys, Arg, His, Asx, and Glx.

Table 2
Summary of the purification strategy for the antioxidant peptide obtained from hydrolysates.

	Purification stage	Fraction	Total peptide (mg)	Purification yield (%)	IC50 (μg/mL)	Purification fold	Extraction yield (%)
CN Trypsin	Hydrolysate	CNH	474.14	100.00	549.46	1.00	23.71
	UF	(<1 kDa)	180.92	38.16	6.95	40.23	9.05
	RP-HPLC	16	15.99	3.372	6.54	377.78	0.80
CB Trypsin	Hydrolysate	CBH	444.19	100.00	946.75	1.00	22.21
	UF	(<1 kDa)	86.58	19.49	6.96	36.65	4.33
	RP-HPLC	3	7.40	1.67	6.13	343.14	0.37
CN Alcalase	Hydrolysate	CNH	406.67	100.00	571.32	1.00	20.33
	UF	(<1 kDa)	106.35	26.15	6.12	32.55	5.32
	RP-HPLC	2	3.69	0.91	7.72	89.63	0.18
CB Alcalase	Hydrolysate	CBH	481.40	100.00	810.73	1.00	24.07
	UF	(<1 kDa)	166.29	34.54	6.81	54.84	8.31
	RP-HPLC	2	5.36	1.11	8.97	134.08	0.27
		3	7.56	1.57	6.36	266.79	0.38

Extraction yield was calculated as a relative percentage of the protein content of fraction obtained by each purification step to total protein content before hydrolysis.
Purification yield was calculated as a relative percentage of the protein content of fraction obtained by each purification step to total protein content for last step purification.
Purification fold divide specific activity of initial hydrolysate to specific activity of each fraction found.

peptides eluted later, while the large polar or hydrophilic peptides eluted much earlier (Zhang, Huang, et al., 2014). Radical-scavenging peptides from active fraction UF-VI were purified using RP-HPLC (Hu et al., 2015). We considered the scavenging potency of radical as the selection criteria of radical scavenging peptides during purification. The UF-VI fraction of trypsin treated hydrolysate was further separated by RP-HPLC on a C_{18} column (21.2 × 150 mm) as shown in Fig. 2 and 21 peaks were selected. Each peak was collected and measured for the antioxidant activity by ABTS (Fig. 2A, B). Due to the large number of interesting samples, a selection was made on the basis of peaks with high score above 50% inhibition and protein content. Among the 21 peaks for UF-VI CNH, 11 peaks showed higher score above 50% inhibition, therefore only 5 peaks were further chosen due to their higher peptide concentrations at the retention time of 23.2, 26.8, 28.7, 30.05 and 33.4 min, and were labeled as 9, 13, 16, 17 and 20, respectively. Mendis, Rajapakse, and Kim (2005) also found neutral or hydrophilic peptide fractions and acidic or hydrophobic peptide fractions in skin gelatin. Further, the fraction 16 and 17 showed the highest radical scavenging activity (76.45 and 80.34%) at a protein concentration of 15.99 and 8.675 mg, respectively.

The UF-VI CBH 2, 3, 18 and 19 fractions exhibited the highest antioxidant activity at 13.0, 13.5, 34.0 and 34.25 min, respectively. However, there was a clear difference noted between UF-VI CBH and UF-VI CNH fractions related to radical scavenging activity, two neutral or hydrophilic peptide fractions (2 and 3) and two acidic or hydrophobic peptide fractions (18 and 19). On the other hand, antioxidant activity fraction UF-VI CBH was observed at middle zone from RP-HPLC peak. These results were in agreement with Shanmugam et al. (2015), who found that the hydrolysates by pepsin first and then trypsin displayed high antioxidant activity with a high peak area at the middle zone of their chromatogram. The most active fractions (fraction 3, 18 and 19) which exhibited high antioxidant activity as well as higher protein concentrations were selected for subsequent peptide identification. Among the selected fractions, fraction 3, 18 and 19 showed higher radical scavenging activity than other fractions (81.6, 86.48 and 87.4%) at a protein concentration of 7.4, 1.76 and 2.415 mg, respectively. With regard to purification folds, the fractions 16 UF-VI CNH (377.78) and 3 UF-VI CBH (343.14) were found higher than other fractions (Table 2).

3.4. Fractionations of UF-VI alcalase CBH and CNH by semi-preparative RP-HPLC

Alcalase hydrolysis had been used for the fractionation of UF-VI CNH and UF-VI CBH same as trypsin hydrolysis. Alcalase hydrolysis enabled protein and peptide cleaved through peptide bonds into large uncharged residues. Hence, enriches of peptides with low molecular weight < 1.2 KDa had high antioxidant activity (Liu et al., 2015).

UF-VI CNH and UF-VI CBH to 31 and 29 peaks were divided. Among the 31 peaks for UF-VI CNH, 6 peaks showed high scores above 50% inhibition, and only 2 peaks with higher antioxidant activity (64.8 and 51.86%) as well as higher peptide concentration (3.69 and 1.32 mg protein) at retention time of 13.2 and 32.43 min (named as 2 and 26), respectively. The 2 peaks were further chosen to MS/MS assay. With regard to UF-VI CBH, 6 peaks showed stronger radical scavenging activity above 50% inhibition. Among all fractions 2 and 3 were chosen to MS/MS assay based on their higher antioxidant activity (55.7 and 78.57%) and peptide concentration (5.358 and 7.56 mg protein). It was observed that alcalase hydrolysis produced hydrophobic and hydrophilic peptides, which had higher antioxidant activity with respect to increased hydrophilic peptides. Consequently, the purification folds values of fraction UF-VI CBH 2 and 3 (266.79 and 134.08) were higher than that (89.63) of UF-VI CNH 2 after the two purification steps, indicating improvement in activity.

3.5. Identification of the antioxidant peptides from fractions trypsin CBH and CNH by LC-MS/MS

The strategy followed to identify the peptides by matching the tandem mass spectra was similar to that used in previous study (Del Mar Contreras, López-Expósito, Hernández-Ledesma, Ramos, & Recio, 2008). LC–MS/MS was able to rapidly resolve peptide components, which provided suitable conditions for the identification of short peptides by fragmentation of long peptide masses formed over fragmentation of short peptides as appropriate collision energy for parent ion fragmentation. It depended on molecular mass and charge of peptide analyzed by LC/MS, while characterization of peptides structure and sequence were analyzed by LC–MS/MS (O'Keeffe & FitzGerald, 2015).

The collected active fractions which had relatively strong antioxidant activity were subjected to LC-MS/MS analysis for the identification of amino acid sequences and molecular mass responsible for the activity, ranging from 3 to 14 amino acid residues in length as shown in Table 3. The major peptides presented in the active fractions UF-VI trypsin CBH were 2, 3, 18 and 19. Fraction 2 (RELEE, MEDNKQ) and 3 (RELEEL) were identified with peptides sequence f18-20 of β-casein and f69-74 αS1 casein, respectively. The result was confirmed by comparison with data from the National Center for Biotechnology Information (NCBI) database. The accurate molecular mass of RELEE and MEDNKQ were 675.30 and 1007.40 Da based on the mass to charge ratio (m/z) of the

Table 3
Peptides identified by MS/MS in fractions collected by preparative RP-HPLC of the UF-VI (<1 KDa) of the buffalo casein hydrolysate (CBH) and bovine casein hydrolysate (CNH) by trypsin and alcalase.

	No. Fraction	RT (min)	Observed mass[M + H]$^+$	MW Calculate	Supposed sequence
Trypsin CNH	9	4.38	297.11	296.1008	DY
		5.38	504.18	503.238	PYPQ
		5.93	1090.32	1089.48	EADTEAEQVV
	13	5.91	1575.78	1574.688	FYDLNPSQSQEFT
		6.22	748.29	747.36	EMPFPK
		6.9	894.88	893.456	AQFLLTTT
	16	6.82	718.26	717.3010	YFYPE
		6.82	408.15	407.169	PYE
		6.81	718.26	717.249	EDYEY
		7.464	731.36	730.33	LFVCYS
	17	7.59	790.40	789.3689	FTFFQT
		7.59	513.29	512.295	GLPNL
		7.87	915.47	914.55	RRKRATQ
		7.848	1084.99	1083.5018	NMSSGVPPAGAP
		7.87	821.37	820.38	SAHNPPGAA
		7.87	527.26	526.27	PPQSV
		7.87	430.21	429.22	PQSV
		7.87	323.16	322.1	GGCS
	20	9.16	1505.79	1504.81	QEPVLGLLVFFPF
		9.16	744.4	743.415	RERRAG
		9.16	753.91	753.29	DFRDDS
		9.35	1124.58	1123.60	QVAPVLNPWT
		9.35	644.28	643.28	EPGDQV
Trypsin CBH	2	3.686	976.36	975.43	FYYEQNL
		3.70	675.30	674.32	RELEE
		4.46	511.20	510.189	GNACF
		4.61	1007.40	1006.43	MEDNKQ
	3	2.87	347.18	346.18	LQS
		3.96	1181.54	1180.612	FALGELAAAAFT
		4.31	1044.49	1043.1	LQEAGTLPSE
		4.31	765.32	764.32	HEGASAGH
		4.52	664.27	663.326	MVSLSQ
		4.91	731.32	730.36	QPPGGYL
		5.96	788.35	787.40	RELEEL
	18	9.39	792.34	791.38	FPEPVFG
		9.53	440.24	439.229	RHQ
		9.53	287.13	286.1277	GPN
		9.53	1528.67	1527.74	GTQEDLNLNSLDLP
	19	9.55	821.36	820.407	LDHATPAP
		9.55	1031.47	1030.50	VDQPHEPPL
		9.55	1132.51	1131.58	TLPDVQYSPL
		9.55	230.14	229.106	GGP
Alcalase CNH	2	1.10	654.27	653.3133	HLQQE
		1.21	466.19	465.1893	CATTA
		1.29	397.18	396.1315	SCTS
		1.71	446.19	445.1995	MAPQ
		1.9	767.26	766.386	TQKDLY
		2.06	485.25	484.239	GHKSG
		2.56	803.32	802.3821	SNSTVPQA
Alcalase CBH	2	1.72	329.18	328.1859	PRG
		2.01	290.16	289.163	TVA
		2.01	190.05	189.075	GN
		2.32	292.15	291.1067	DAS
		2.55	635.26	634.3174	STLLDS
	3	1.27	843.23	842.483	RDRRLQ
		1.65	389.25	389.19	TAAAG
		2.00	960.34	959.517	LLTDLALDS
		2.31	292.12	291.106	DAS
		2.31	797.37	796.36	YQTLDSA
		2.55	635.29	634.299	VMTDLG
		2.78	726.34	725.468	PLLLLAS
		3.10	532.25	531.326	LLSLS
		3.72	389.20	388.1958	EQL
		4.95	1177.54	1176.6642	LLLLYLDDSL
		5.15	690.3	689.32	LDSENL
		5.32	796.4200	795.52	PVNLLLK
		5.32	372.17	371.1627	HCl

molecular ion peak ([M+H$^+$]). While fraction 18 and 19, the amino acid sequences GP, HQ and PL were found both within the sequences, RHQ, GPN, GGP and last letter sequences in VDQPHEPPL, TLPDVQYSPL. The sequences (GP, HQ and PL) distinguished β-Casein, α_{s1} Casein and amino acids. Glutamine (Q), proline (P), histidine (H), glycine (G) and leucine (L) were also presented in these peptides, which were all considered to be important amino acids for peptide antioxidant activity (Leeb et al., 2014).

Different from UF-VI CNH sample 9, 13, 16, 17 and 20 peptides PYPQ, EMPFPK, YFYPE, PQSV and PPQSV were identified with data comparison from NCBI as a f194-197 of β-Casein (F9) f122-128 of β-Casein (F13), f158-163 of α_{s1}-Casein (F16), f174-177 of β-Casein (F17) and f173-177 of β Casein (F17), respectively. Peptides containing valine (V), glutamic acid (E), lysine (K) and glutamine (Q) as C-terminal residue might have contribution for peptide antioxidant activity (Zhang, Xiong, Chen, & Zhou, 2013) which demonstrated higher radical scavenging potential. In addition, to the known peptides mentioned above, a number of other peptides identified in fractions might have contribution to the radical scavenging activity as a result of structure-activity of amino acids (Table 3). Also, the other identified peptides showed their C-terminal residues played a predominant role in the inhibition of the free radicals significantly through the donation of hydrogen atoms mechanism. More than 50% of the identified sequence chain peptides of the residues in all fractions composed of phenylalanine (F), proline (P), threonine (T), leucine (L), glutamine (Q), alanine (A) and glycine (G), which may lead to a higher antioxidant activity (Power et al., 2013).

3.6. Identification of the antioxidant peptides from fractions alcalase CBH and CNH by LC–MS/MS

Previous studies had shown that amino acids sequences of antioxidant peptides derived from alcalase hydrolysate were identified by MS/MS (Cheng et al., 2010). Identification of amino acid sequences of UF-VI CBH fraction 2 and 3, UF-VI CNH fraction 2 which had relatively strong antioxidant activity, were subjected to LC–MS/MS as shown in Table 3. Antioxidant peptides separated from fractions of alcalase CNH and CBH were hydrophilic characterized and obtained at RP-HPLC zone. Recently, characterization of antioxidant peptides indicated that antioxidant activity mainly depended on the bioactive amino acids acting as electrons or hydrogen donors (Sila & Bougatef, 2016) and their sequence position as well as quantity (Wattanasiritham, Theerakulkait, Wickramasekara, Maier, & Stevens, 2016).

The peptides in UF-VI CNH fraction 2 consisting of hydrophobic amino acids coupled with proline (P), glutamine (Q), glutamic acid (E) and serine (S) which presented in their peptides had contributed to strong antioxidant activity in peptides sequence

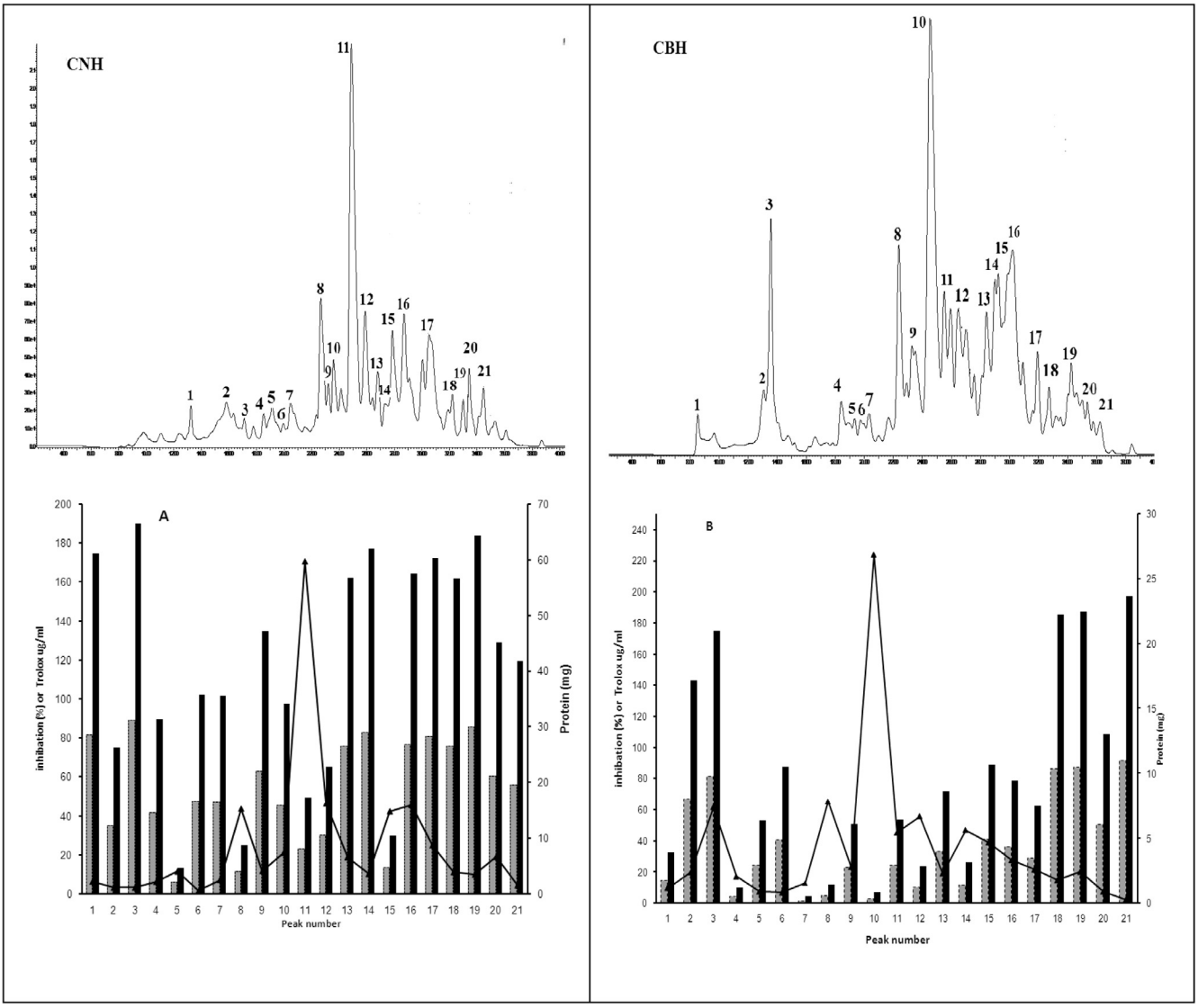

Fig. 2. RP-HPLC chromatography profile of active fraction UF-VI (MW < 1 kDa) from (A) bovine casein hydrolysate (CNH) by trypsin, (B) buffalo casein hydrolysate (CBH) by trypsin, (C) bovine casein hydrolysate (CNH) by alcalase and (D) buffalo casein hydrolysate (CBH) by alcalase. Antioxidant activity as percent of inhibition (Gray columns) and equivalent Trolox (black columns) of each fraction isolated by RP-HPLC and protein content (lines).

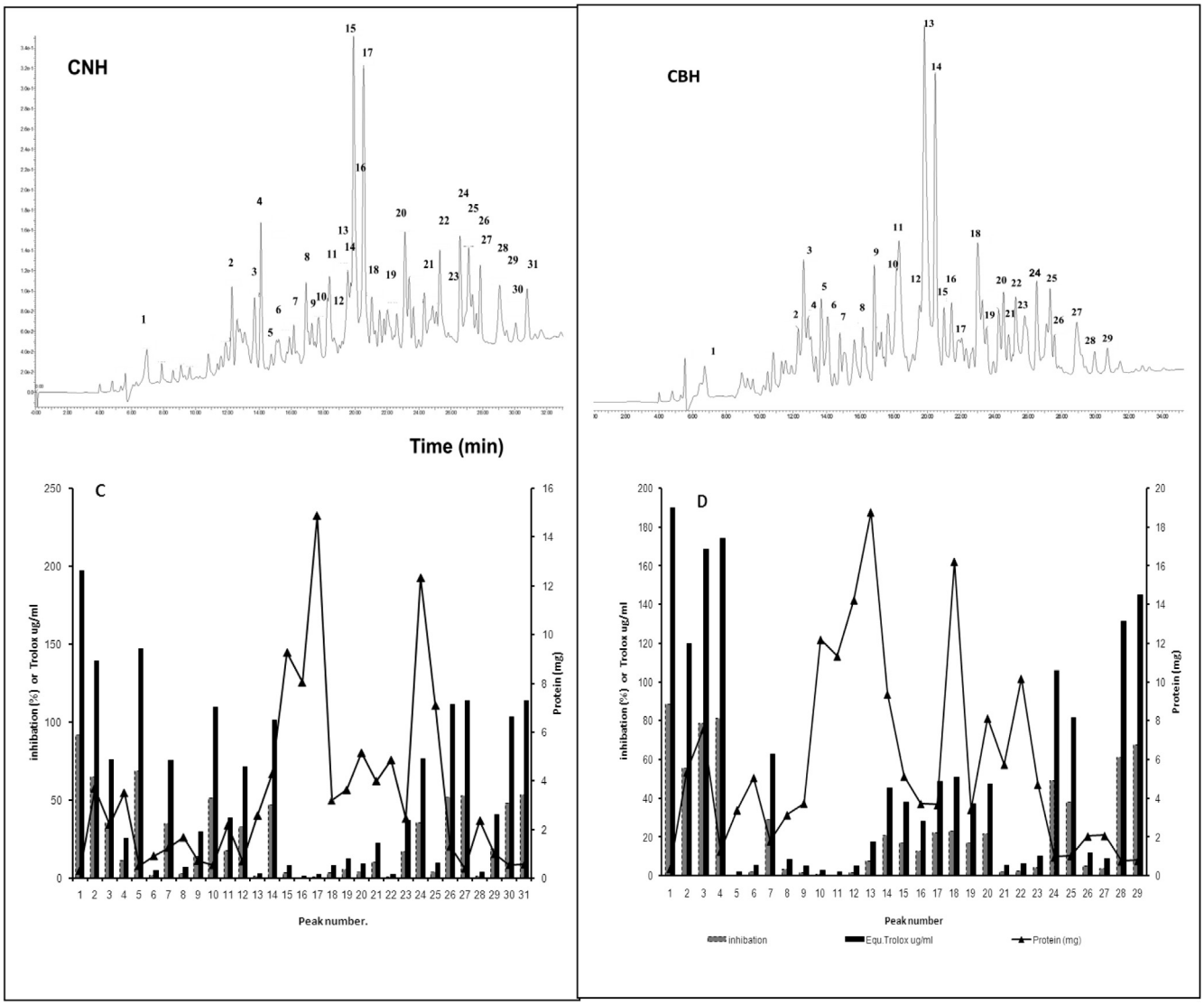

Fig. 2 (*continued*)

(De Gobba et al., 2014). For instance, HLQQE, SCTS, SNSTVPQA and MAPQ, had hydrophobic amino acids such as alanine (A), glycine (G), threonine (T), cysteine (C) and tyrosine (Y) at the C- and N-terminal that contributed to strong antioxidant activity of peptides. Cheng et al. (2010) and Zhang, Huang, et al. (2014) had also reported that antioxidant peptides consisted of hydrophobic residues in their sequences CATTA, GHKSG and TQKDLY.

UF-VI CBH fractions 2 and 3 consisted of low MW peptides. In particular, fraction 2 (<634 Da) exhibited stronger antioxidant activity than large MW peptides (Zhang, Mu, et al., 2014). TVA identified peptide as sequences f163-165 of K-casein by NCBI, in addition to hydrophobic amino acid alanine (A) contained, which played a crucial role in antioxidant activity (Jung, Kim, & Kim, 1995). Also, that peptide sequence VA at the C-terminal in peptide WHSVA derived from β-Lg I (Le Maux, Nongonierma, Murray, Kelly, & FitzGerald, 2015) possessed strong antioxidant activity. Moreover, the position of threonine (T) at N-terminus displayed higher activity (Le Maux et al., 2015). Hence, our findings suggested that the presence of TVA peptide in fraction 2 contributed to the antioxidant activity of UF-VI CBH fraction.

Fraction 3 demonstrated stronger antioxidant activity than fraction 2 and contained peptides with leucine (L) in their sequence. Some studies had also found leucine as a common amino acid in the sequence could be the reason for high radical scavenging potential (Umayaparvathi et al., 2014). Repetitive sequence of LL was found in five peptides PVNLLLK, LLLLYLDDSL, LLSLS, PLLLLAS and LLTDLALDS (Jin, Liu, Zheng, Wang, & He, 2016). Peptide EQL was identified at sequences f103-105 and f125-127 of α_{s1}-casein, f132-134 and f141-143 α_{s2}-casein by NCBI. Sila and Bougatef (2016) demonstrated that the high amount of glutamic acid (E) played an important role in high antioxidant activity. In general, glutamic acid had contribution in antioxidant activity within removal of hydrogen peroxide producing the oxidized glutathione (Nimalaratne, Bandara, & Wu, 2015; Power et al., 2013).

3.7. The proposed model for interaction between active peptide with ABTS

Antioxidant activity was based on the reduction of ROS (reactive oxygen species) in the biological system and lipid peroxidation in foods. Likewise in chemical reaction, radical cation $ABTS^{\cdot+}$ (blue-green) converted to stable ABTS (colorless) by donating electron donors or hydrogen. The proposed mechanism pathways of antioxidant peptides were based on hydrogen transfer or oxidation of amino acids in peptides to $ABTS^{\cdot+}$, then stabilizing ABTS as the final product. The peptides RELEE, EQL and TVA were selected to react

with ABTS radical to provide a new mechanistic insight to understand the possible antioxidant mechanism as shown in Fig. 3.

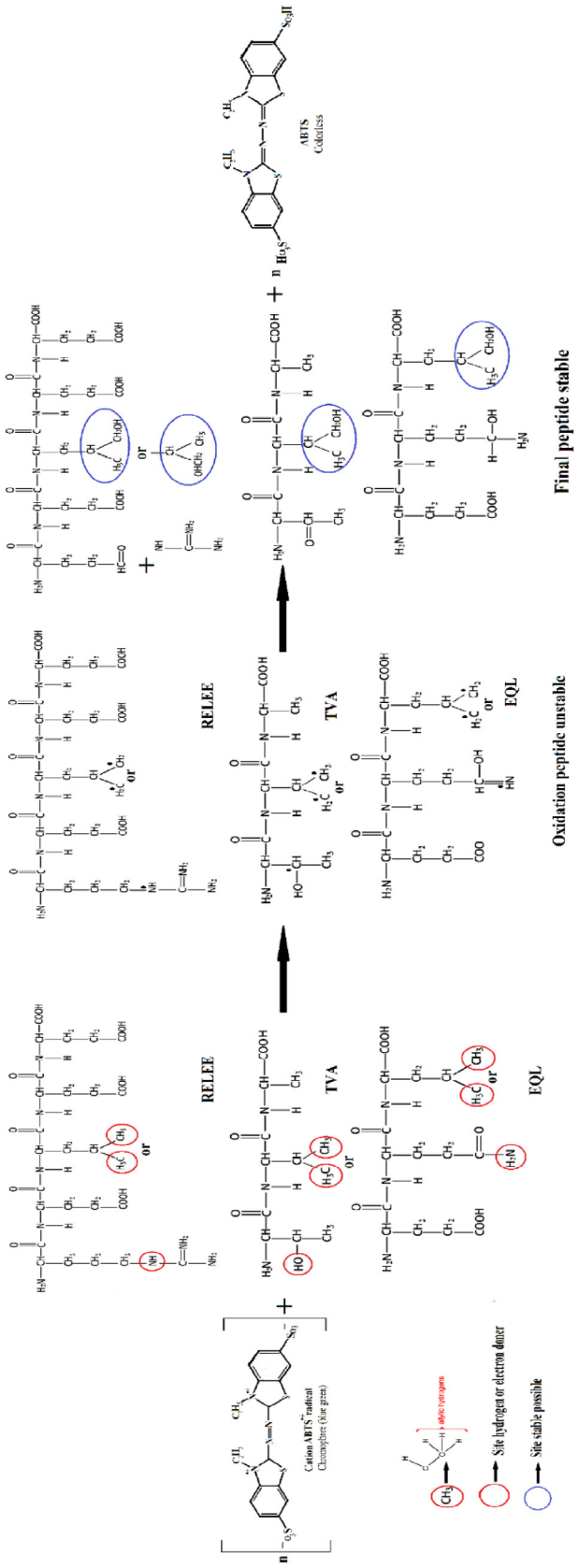

Fig. 3. Proposed reaction pathways of peptides RELEE, TVA and EQL quenching ABTS cation radical.

Hydrophobic amino acids argentine (R), leucine (L) and valine (V), had an abundance of hydrogen in methyl group, which was an allylic hydrogen that was very active and easily abstracted by free radical (Jian-Ming, Peter, Qizhi, & Changyi, 2010) coupled with an active hydrogen atom in hydroxyl group at threonine (T). Stadtman and Levine (2003) reported that oxidation of arginine, leucine and threonine generated glutamic-semialdehyde, 3-, 4-, 5-hydroxyleucine and 2-amino-3-keto butyric acid, respectively. Additionally, NH_2 or NH groups might have also contributed in donating active hydrogen by oxidation of glutamine (Q). As described in above paragraph, amino acids played important roles in the mechanism of antioxidant. Consequently, every peptide possesses at least two amino acids to demonstrate its antioxidant potential. However, no previous studies had elucidated a mechanism of antioxidants with ABTS radical. Therefore, the proposed mode could be used to explain regulation of redox potential in a biological and food systems.

4. Conclusion

Results from the present study had demonstrated that the antioxidant activity from CBH effectively scavenged ABTS radicals as compared to the CNH. Alcalase hydrolysis was more suitable for CB compared to CN. In addition, fractionation of hydrolysates increased the antioxidant properties especially with a molecular weight less than 1 KDa that consisted of hydrophobic and specific hydrophilic amino acids which further had effect on the peptide antioxidant activity. The total protein contents of UF-VI fraction was found higher among the lower molecular (<1 kDa) fractions as compared to the other fractions. A novel peptide was identified from CB and CN hydrolysates, which showed high antioxidant activity and could be potentially used in pharmaceuticals or in formulating functional foods as natural antioxidant.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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