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Isolation and identification of alcohol dehydrogenase stabilizing peptides from Alcalase digested chicken breast hydrolysates

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ABSTRACT

The effect of chicken hydrolysates (CHs) on alcohol dehydrogenase (ADH) stability was investigated, together with further bioactivity-oriented isolation and identification of CHs. A total of 82 peptides were identified using mass spectrometry in tandem after consecutive separation by size-exclusion chromatography and high-performance liquid chromatography. The identified peptides were then subjected to *in silico* gastrointestinal digestion and 154 peptides were generated. The potential bioactivity, safety and applicability of the peptides were assessed using multiple predictive programs. A total of 21 among the 154 peptides were predicted to be potentially active with applicability. Four peptides (DPQYPPGPPAF, QKPVL, KPC, and APGH) obtained after *in silico* digestion were synthesized and validated their activity. Results showed that DPQYPPGPPAF, KPC, and APGH could stabilize ADH in a dose-dependent manner. This study further indicated that chicken hydrolysate could be a novel functional food ingredient in facilitating alcohol metabolism and protection against alcoholic liver injury.

1. Introduction

In the last few decades, alcohol abuse has become a global problem for human health, with alcoholic liver disease (ALD) being the most significant (Dolganiuc, 2009; Massey & Arteel, 2012). In human body, more than 90% of ingested alcohol is metabolized in the liver. The liver consequently is the main organ that suffers from alcoholic toxicity (Louvet & Mathurin, 2015; Zhao, Huo, Qian, Ren, & Lu, 2017). It is generally believed that alcohol-induced liver injury is originated from high concentration of alcohol together with its metabolites including acetaldehyde and reactive oxygen species (ROS) (Massey & Arteel, 2012). Thus, rapid elimination of such toxic factors can reduce their impairment on liver tissue and ameliorate liver injury.

In normal mammal, about 90% of ingested alcohol is metabolized through the alcohol dehydrogenase (ADH, EC 1.1.1.1) dominated pathway (Cederbaum, 2012; Louvet & Mathurin, 2015). In this pathway, ADH catalyzes alcohol into toxic acetaldehyde, using β-nicotinamide adenine dinucleotide (NAD^+) as a cofactor, while aldehyde dehydrogenase (ALDH) further oxidizes acetaldehyde to non-toxic acetate. Hepatic ADH activity is considered the major factor that limits alcohol metabolism *in vivo* (Haseba, Tomita, Kurosu, & Ohno, 2003; Plapp, Leidal, Murch, & Green, 2015; Raj, Ramaswamy, & Plapp, 2014). On one hand, excessive substrate and product can inhibit ADH activity (Crabb, Bosron, & Li, 1983) whereas acute alcohol exposure tend to suppress liver ADH rather than ALDH (Xiao, Zhou, Zhao, Su, & Sun, 2018; Zhang et al., 2019). Moreover, heavy and chronic alcohol ingestion could also lead to the activation of alcohol metabolism through the microsomal ethanol oxidation system (MEOS). MEOS is an oxygen-dependent pathway, through which massive reactive oxygen species (ROS, mainly $OH^{\cdot}$ and $O_2^{-\cdot}$) are produced, resulting in oxidative stress and thus injury in the liver (Teschke, 2018). As a result, the stabilization or activation of ADH could not only facilitate the elimination of alcohol and its metabolites from blood, but also reduce alcohol metabolism through the MEOS pathway and eventually reduce the impairment on liver tissue.

Bioactive peptides derived from food proteins have been considered

Abbreviations: ACN, acetonitrile; ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; CHs, chicken hydrolysates; FA, formic acid; GI, gastrointestinal; MEOS, microsomal ethanol oxidation system; MW, molecular weight; NAD^+, β-nicotinamide adenine dinucleotide; ROS, reactive oxygen species; RP-HPLC, reverse phase high-performance liquid chromatography; SEC, size-exclusion chromatography

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natural alternatives to promote human health (Nongonierma & FitzGerald, 2016; Sultan, Huma, Butt, Aleem, & Abbas, 2018). Such bioactive peptides can exert various bioactivities after being released from parent protein, including antioxidant, antihypertensive, anti-diabetic, anti-inflammatory, antimicrobial, mineral binding, hepato-protective effect, etc., in addition to nutritional value (Mora, Gallego, & Toldrá, 2018). To date, peptides derived from various proteins, including corn (Yamaguchi, Nishikiori, Ito, & Furukawa, 1997; Yu, Li, He, Huang, & Zhang, 2013; Zhang, Zhang, & Li, 2012), Chum Salmon skin collagen (Liang et al., 2014; Lin et al., 2012), mushroom (Zhao et al., 2017), and chicken meat protein (Lin et al., 2017), have been reported to facilitate alcohol metabolism. However, few researches have been focused on the isolation and identification of novel peptides exerting ADH stabilizing activity.

Chicken breast is consumed worldwide as a perfect source of high quality protein. Chicken protein is considered a good source of bioactive peptides due to the well-balanced amino acid composition (Cui, Zhou, Zhao, & Yang, 2009; Sun, Pan, Guo, & Li, 2012). Our previous study indicated that CHs obtained through Alcalase digestion could activate ADH, facilitate alcohol elimination and ameliorate alcohol-induced liver injury in mice (Xiao et al., 2018). As a continuous work, the objective of this study was to isolate and identify novel peptides exerting ADH stabilizing activity from CHs using *in silico* and *in vitro* analysis.

2. Materials and methods

2.1. Materials

Chicken breast muscle (CBM) was obtained from a local market 24 h after slathering (Valencia, Spain). Alcalase 2.4L, ADH (from Saccharomyces cerevisiae) and β-nicotinamide adenine dinucleotide (NAD^+) were obtained from Sigma Aldrich, Co. Ltd (St. Louis, MO, USA). Sodium pyrophosphate and sodium phosphate were purchased from Panreac Química, S.A.U. (Barcelona, Spain). All chemicals and reagents used were of analytical or chromatographic grade.

2.2. Preparation of CHs

CHs was prepared according to our previous study with minor modification (Xiao et al., 2018). In brief, CBM was minced and 10 g sample were mixed with 50 mL tris-HCl buffer (50 mM, pH 8.0) before the addition of 0.5% (w/w, protein basis) Alcalase 2.4 L. The mixture was continuously incubated and stirred at 55 °C for 8 h in the digestor (Carousel 6 Plus Reaction Station, Radleys, UK) and subsequently heated in boiling water for 10 min to terminate the hydrolysis reaction. The hydrolysates were centrifuged at 10000 rpm for 20 min at 4 °C after being cooled to room temperature. The supernatant was filtered through glass wool, and 3 volumes of ethanol were added to precipitate the proteins (4 °C, 20 h). After centrifugation at 10000 rpm for 20 min, the ethanol was removed in a rotatory evaporator. The sample was then freeze-dried and stored at −20 °C until further analysis.

2.3. Effect of CHs on ADH stability

The stability of ADH was studied pre-incubating different concentration of CHs (0, 0.01, 0.1, 1 and 5 mg/mL, 50 μL) with the same volume of ADH (0.2 U/mL dissolved in 10 mM PBS, pH 7.4) at 37 °C for 0, 15, 30, 60, 90 and 120 min, respectively, before the detection of enzyme activity. ADH activity was determined by adding 150 μL of pre-incubated reaction reagent (containing 22.4 mM sodium pyrophosphate buffer, 3.3% ethanol and 7.8 mM NAD^+). The resultant β-nicotinamide adenine dinucleotide hydrate (NADH) was recorded at 340 nm every 10 s for 10 min under 37 °C incubation using a CLARIOstar microplate reader (BMG LABTECH, Ortenberg, Germany). The reaction curve was fitted and the first derivative of the fitting curve at 0 min was calculated as the initial reaction rate. The original ADH activity was considered 100%, distilled water instead of CHs was used as a negative control. Relative activity was calculated according to the following formula:

$$\text{Relative activity (\%)} = V_s / V_0 \times 100\% \quad (1)$$

Here, V_0 was the original ADH activity, while V_S was that of the negative control or tested samples. All the determinations were carried out in triplicates.

2.4. Fractionation of CHs using size-exclusion chromatography (SEC)

A total 1 g of CHs was dissolved in 5 mL distilled water and filtered using a 0.45 μm syringe filter before being loaded into a Sephadex G25 column (2.5 × 65 cm; Amersham Biosciences, Uppsala, Sweden). The peptides were eluted using 0.01 N HCl at 4 °C with a flow rate of 5 mL/20 min. The fractions of 5 mL were collected and monitored at 214, 254 and 280 nm, respectively, using an Agilent UV spectrophotometer (Agilent 8453, Agilent Technologies, Palo Alto, CA, USA). Fractions were then pooled every 25 mL from 101 mL to 600 mL of elution volume to get 20 fractions namely F1-F20. These 20 fractions were lyophilized and stored at −20 °C until further analysis.

2.5. Isolation using reversed-phase high-performance liquid chromatography (RP-HPLC)

The most active fraction obtained from SEC fractionation was further isolated using an Agilent 1100 HPLC system (Agilent Tech., California, USA) with a Symmetry C18 column (250 × 4.6 mm, 5 μm; Waters Co. Milford, MA, USA). The mobile phases consisted of solvent A: 0.1% v/v trifluoroacetic acetic acid (TFA) and solvent B: 0.085% v/v TFA in acetonitrile (ACN). Peptides were diluted using the following gradient: 0% B from 0 to 2 min, linearly increasing to 30% B at 50 min, 60% B at 60 min and 100% B at 65 min under a flow rate of 1 mL/min. Absorbance was monitored at 214 nm and fractions were collected every 3 min to get a total of 22 fractions namely f1-f22. These fractions were freeze-dried and stored at −20 °C until further analysis.

2.6. Identification of peptides by nESI–LC–MS/MS

The most active fractions from HPLC isolation were further analyzed using an Eksigent nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) tandem nanoelectrospray ionization source-quadrupole-time-of-flight (nanoESI-Q-ToF) TripleTOF® 5600 system (AB Sciex Instruments, MA, USA). The lyophilized sample was re-dissolved in distilled water and concentrated using Zip-Tip C18 (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions. After drying, samples were resuspended in 50 μL of 0.1% TFA in 2% ACN. A total 2 μL of sample was loaded onto an Eksigen trap column (3 μm C18-CL, 350 μm × 0.5 mm) for 5 min at 3 μL/min before injected into the analytical column (3 μm C18-CL, 75 μm × 123 mm; Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A: 0.1% v/v formic acid (FA) in water and solvent B: 1% FA in acetonitrile (ACN). Peptides were diluted linearly from 5% to 35% solvent B over the first 20 min, and then from 35% to 65% solvent B for 10 min under a flow rate of 0.3 μL/min at 30 °C.

The flow from the LC system was directly injected into the mass spectrometry system and ionized applying 2.8 kV. The Q-ToF was operated in positive polarity and information-dependent acquisition mode. MS1 scan was acquired from 350 to 1250 *m/z* for 250 ms, while MS2 scan was required from 100 to 1500 *m/z* for 50 ms on 50 of the most intense ions charging from 1 to 5. Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s.

The database search of peptides was performed using the Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA; http://www.matrixscience.com), and Mascot search engine with a significance

threshold $p < 0.05$ using Chordata taxonomy, none enzyme digestion, and Uniprot database. The tolerance on the mass measurement was 0.3 Da for MS and 100 ppm for MS/MS. On the other hand, ProteinPilot v 4.5 software from (ABSciex) was used to search in NCBInr database with the following parameters: no enzyme specificity, no taxonomy restriction, and the search effort set to through.

2.7. In silico analysis and screening of the bioactive peptides

The potential stability of peptides during gastrointestinal (GI) tract was assessed using the ExPASy PeptideCutter tool (http://web.expasy.org/peptide_cutter/). Pepsin pH 1.3 and pH > 2.0 (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were chosen as digestion enzymes (Garcia-Vaquero, Mora, & Hayes, 2019).

The potential bioactivity of all the identified peptides and their possible fragments after *in silico* GI digestion were predicted using Peptide Ranker software (http://distilldeep.ucd.ie/PeptideRanker/). Peptides were scored from 0 to 1 and higher score means higher probability to be bioactive (Tu et al., 2019).

The potential peptide allergenicity was predicted using the AllerTOP v. 2.0 software (http://www.ddg-pharmfac.net/AllerTOP/index.html) (Polikovsky et al., 2019).

Peptide toxicity and physicochemical properties (i.e., hydrophobicity, amphipathicity, steric hindrance, and pI) were studied using ToxinPred software (http://crdd.osdd.net/raghava/toxinpred/) (Gupta et al., 2013).

2.8. Peptide synthesis

Peptides were synthesized by the Peptide Synthesis facility of the Department of Experimental and Health Sciences in the Universitat Pompeu Fabra (Barcelona, Spain) at the highest purity certified using liquid-chromatography mass spectrometry (LC-MS) analysis. The synthesized peptides were stored at −80 °C until used.

2.9. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS 13.0 for Windows, SPSS Inc, Chicago, IL). The homogeneity of the data was tested before mean comparison applying the one-way analysis of variance (ANOVA) following Duncan's test. Difference of $p < 0.05$ was considered statistically significant. Standard deviation (SD) was shown as error bars on the figures.

3. Results and discussion

3.1. Effect of CHs on ADH stability

Despite the critical role of ADH on alcohol metabolism, the enzyme activity can decrease during the metabolism, because of the loss of enzyme stability (Dumont et al., 2018). A previous study demonstrated that CHs could activate ADH *in vitro* and *in vivo* although the exact activation mechanism is still not clear (Xiao et al., 2018). In this research, we firstly investigated the effect of CHs on ADH stability at 37 °C. As shown in Fig. 1, ADH activity decreased sharply with time of incubation. The relative activity decreased to about 10% after 60 min of incubation, being completely inactivated at 120 min. However, higher ADH activity remained when incubating with CHs higher than 0.1 mg/mL. When CHs reached 5 mg/mL level, ADH kept almost 100% of its activity. Results indicated that ADH was inactivated along with incubation, while the existence of CHs could stabilize ADH and maintain its activity at a higher level. Various methods to improve enzyme stability were reported (i.e. using additives, chemical modification of enzyme, and enzyme immobilization), but using additives (i.e. BSA, casein, glutaraldehyde, and glycerol) was the most popular (Iyer & Ananthanarayan, 2008). Such additives could bind to special active site and thereby stabilizing the conformation of the enzyme (Brougham & Johnson, 1981; Iyer & Ananthanarayan, 2008).

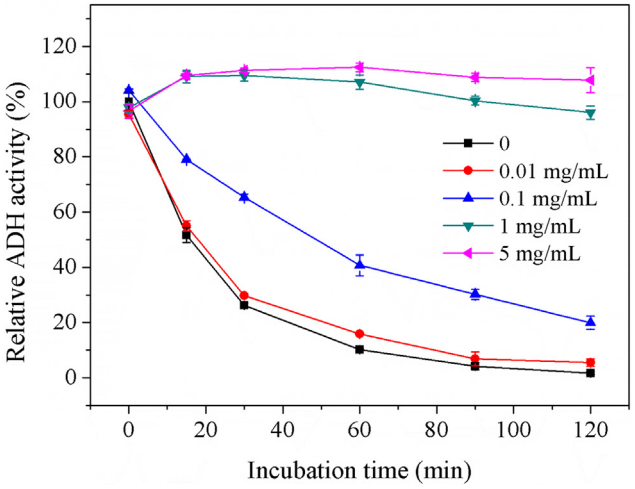

Fig. 1. Effect of different concentration of CHs (0–5 mg/mL) on ADH activity during different incubation times (0–120 min) at 37 °C.

3.2. Fractionation of CHs by SEC

To further understand the exact peptide fractions that may stabilize ADH, CHs was firstly fractionated and concentrated using a Sephadex G25 column that separates according to their molecular weight (MW). A total of 1 g (dissolved in 5 mL distilled water) of CHs was loaded to the column and 5 mL fractions were collected for the determination of chromatographic profile and pooled every 25 mL from 101 mL to 600 mL of elution volume to get a total of 20 fractions namely F1-F20, which were lyophilized and re-dissolved to 0.1 mg/mL before being subjected to ADH stabilizing assay. As can be seen in Fig. 2A, CHs can be crudely separated into 8 fractions (1: 120–170 mL, 2: 120–250 mL, 3: 250–275 mL, 4: 275–325 mL, 5: 325–380 mL, 6: 380–420 mL, 7: 420–500 mL and 8: 500–600 mL). Regarding activity, as shown in Fig. 2B, ADH activity decreased to about 15% after 1 h incubation at 37 °C (Control), however, it retained significantly higher activity when incubated with 0.1 mg/mL of peptide fractions, with F4 being the most active fraction which maintained ADH activity at 66.5%, which was 4 times higher than control. Moreover, fractions with higher MW (F2-F5) tended to exert better activity than those with lower MW (F6-F20). Although peptides with relatively lower MW were generally considered to be more active (Yu et al., 2013), relatively longer peptide chain could contribute to form a favorable spatial conformation, further resulting in stronger interaction between peptides and enzyme that influenced the preservation of its activity (Ma, Wu, & Li, 2018). Results indicated that the SEC isolation was effective, and the most active fraction F4 was further subjected to RP-HPLC separation.

3.3. RP-HPLC separation

RP-HPLC is one of the most common methods used for peptide separation and isolation, which can also provide information about differences in hydrophobicity between fractions (Moayedi et al., 2018). The most active fraction F4 from SEC separation was further injected into the RP-HPLC system. Fractions were collected every 3 min, and a total of 22 fractions were obtained after 65 min. Fractions were lyophilized and re-dissolved in distilled water before being subjected to ADH stabilizing assay. Results are shown in Fig. 3. As can be seen, fraction f20 exerted significantly better activity than the other fractions, as about 70% ADH activity maintained after 1 h incubation. Fraction f20 was relatively hydrophobic as it eluted at 58–60 min with

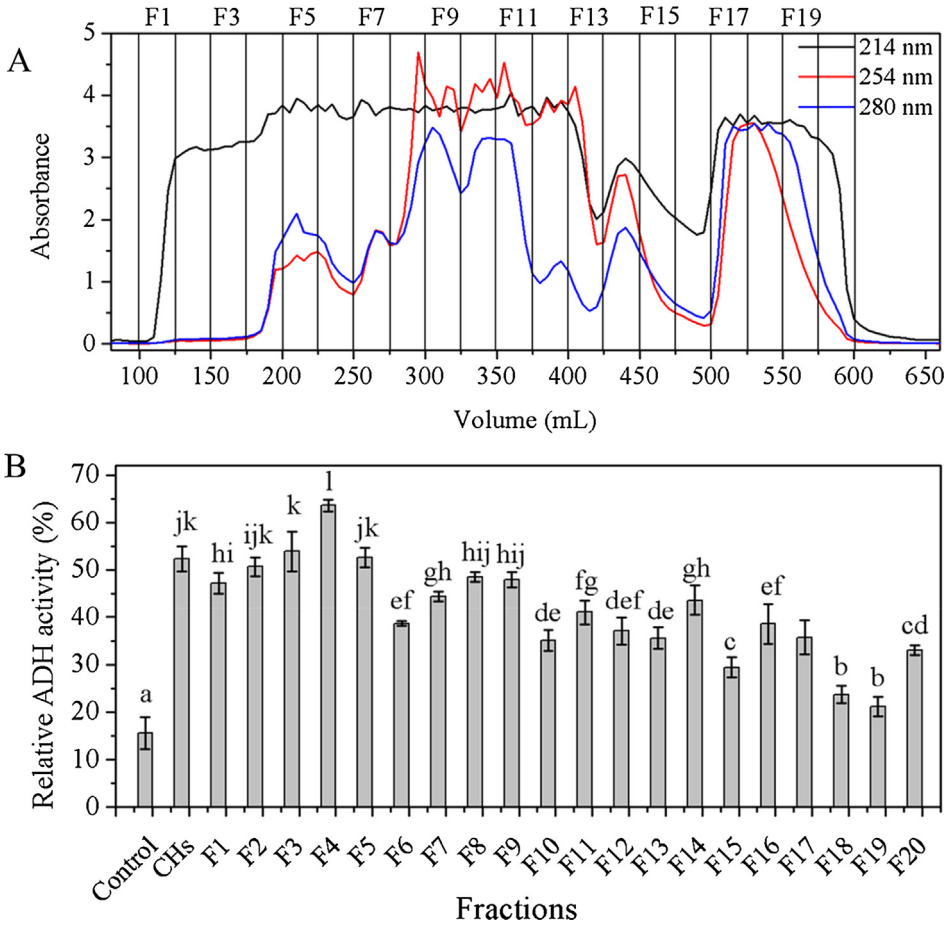

Fig. 2. The SEC fractionations of CHs. (A) The SEC profile of CHs separated by a Sephadex-G25 column and the peptide fractions collected; (B) Effect of SEC fractionation (0.1 mg/mL) on ADH activity (60 min incubation at 37 °C).

32.4–36% ACN. Hydrophobicity was considered an important factor that may contribute to peptide-enzyme interaction that may further affect structure and activity of the enzyme (Pelay-Gimeno, Glas, Koch, & Grossmann, 2015). These results also supported our previous study reporting that hydrophobicity is an important factor that contributes to ADH stabilizing activity. The most active fraction f20 was subsequently subjected to peptide identification.

3.4. Identification of peptides and analysis of their potential bioactivity and applicability

The most active fraction obtained after RP-HPLC separation (f20)

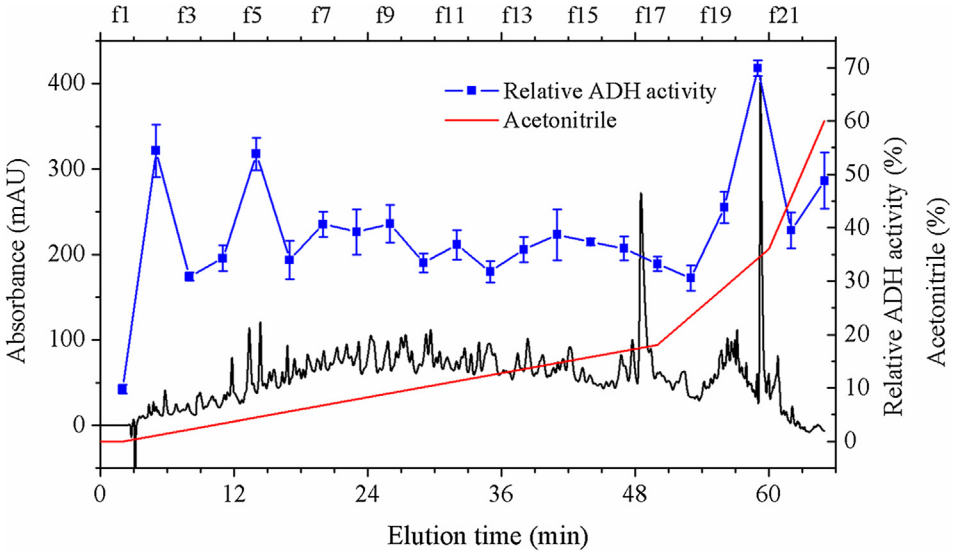

Fig. 3. RP-HPLC profile of the most active fraction F4 obtained from SEC fractionation together with the effect of each HPLC fraction on ADH activity (60 min incubation at 37 °C).

Table 1
Peptides identified from fraction f20 after RP-HPLC isolation and the prediction of their potential bioactivity.

No.	Parent Protein	Observed M/Z	Charge	Calculated MW (Da)	P0	Peptide Sequence	Pf	Peptide Ranker Score
1	Creatine kinase	439.73	2	877.45	A	AVGAVFDIS	N	0.22
2	Creatine kinase	473.74	2	945.48	K	DLFDPVIQ	D	0.31
3	Creatine kinase	949.41	1	948.41	S	AEEEFPDL	S	0.33
4	Creatine kinase	496.27	2	990.52	Q	MVVDGVKLM	V	0.25
5	Creatine kinase	1061.51	1	1060.51	K	DLFDPVIQD	R	0.18
6	Creatine kinase	537.79	2	1073.58	F	KDLFDPVIQ	D	0.27
7	Creatine kinase	600.28	2	1198.54	T	GVDNPGHPYIM	T	0.69
8	Creatine kinase	724.38	2	1446.75	A	AVGAVFDISNADRL	G	0.39
9	Creatine kinase	742.36	2	1482.70	K	LEQNQPIDDMIPA	Q	0.32
10	Creatine kinase	510.59	3	1528.76	I	DDHFLFDKPVSPL	L	0.75
11	Creatine kinase	765.38	2	1528.76	L	IDDHFLFDKPVSP	L	0.46
12	Creatine kinase	821.92	2	1641.84	L	IDDHFLFDKPVSPL	L	0.71
13	Creatine kinase	927.93	2	1853.86	L	DDVIQTGVDNPGHPFIM	T	0.43
14	Creatine kinase	953.96	2	1905.91	F	DISNADRLGFSEVEQVQ	M	0.10
15	Creatine kinase	961.47	2	1920.94	L	RDKETPSGFTLDDVIQT	G	0.10
16	Titin	562.33	2	1122.64	D	IPGPPTGPIKF	D	0.88
17	Titin	581.79	2	1161.57	K	FPFDVPSEPK	N	0.58
18	Titin	585.32	2	1168.63	K	EDLQKPVLDL	K	0.19
19	Titin	589.85	2	1177.68	K	VIDVPGPVRNL	E	0.45
20	Titin	624.86	2	1247.71	F	DVPGPVLDLKPV	V	0.47
21	Titin	640.31	2	1278.61	K	DTFTTPGPPYAL	A	0.29
22	Titin	641.80	2	1281.60	R	DPQYPPGPPAFP	K	0.85
23	Titin	662.86	2	1323.72	E	VNVLDKPGPPAAF	D	0.42
24	Titin	712.87	2	1423.75	R	ATGNPNPDIVWLK	N	0.60
25	Titin	719.86	2	1437.70	A	RDPQYPPGPPAFP	K	0.76
26	Titin	755.37	2	1508.74	Y	ARDPQYPPGPPAFP	K	0.72
27	Titin	800.92	2	1599.85	L	RIPVVLPEDEGIYT	A	0.15
28	Enolase	452.75	2	903.49	E	FMVLPVGAA	S	0.30
29	Enolase	1053.53	1	1052.51	L	DNHEALELL	K	0.23
30	Enolase	704.78	2	1407.55	S	IEDPFDQDDWE	A	0.28
31	Enolase	798.89	2	1595.76	E	GGFAPNILDNHEALE	L	0.37
32	Enolase	847.95	2	1693.89	P	VPAFNVINGGSHAGNKL	A	0.60
33	Enolase	888.92	2	1775.83	M	VSHRSGETEDTFIADL	V	0.10
34	Enolase	635.68	3	1904.03	V	LPVPAFNVINGGSHAGNKL	A	0.70
35	Enolase	969.46	2	1936.92	K	YGKDATNVGDEGGFAPNIL	E	0.32
36	Enolase	1069.03	2	2136.05	K	AKYGKDATNVGDEGGFAPNIL	E	0.28
37	Actin	483.77	2	965.54	K	HLDIPKML	D	0.66
38	Actin	660.32	2	1318.63	A	GFAGDDAPRAVFP	S	0.67
39	Actin	688.83	2	1375.65	K	AGFGGDDAPRAVFP	S	0.69
40	Actin	691.83	2	1381.65	L	DLAGRDLTDYLM	K	0.36
41	Actin	900.90	2	1799.80	N	NVMSGGTTMYPGIADRM	Q	0.18
42	Calsequestrin	818.35	1	817.35	I	DPDDFPL	L	0.91
43	Calsequestrin	601.32	2	1200.62	F	DQIDDEIKLI	G	0.25
44	Calsequestrin	711.36	2	1420.71	F	DQIDDEIKLIGY	F	0.30
45	Calsequestrin	826.91	2	1651.82	L	NFPTYDGKDRVIDL	N	0.23
46	L-lactate dehydrogenase	722.89	2	1443.78	L	TLVDVVEDKLKGE	M	0.06
47	L-lactate dehydrogenase	848.90	2	1695.79	V	GEHGDSSVPVWSGVNVA	G	0.35
48	L-lactate dehydrogenase	1230.67	2	2459.33	H	NKISVVGVGAVGMACAISILMKDLA	D	0.86
49	Triosephosphate isomerase	895.46	2	1788.90	L	AYEPVWAIGTGKTATPQ	Q	0.22
50	Triosephosphate isomerase	508.62	3	1522.85	S	LKPEFVDIINAKH	–	0.28
51	Triosephosphate isomerase	508.95	3	1523.83	M	NGDKKSLGELIHTL	N	0.39
52	Triosephosphate isomerase	537.63	3	1609.88	A	SLKPEFVDIINAKH	–	0.40
53	Glyceraldehyde-3-phosphate dehydrogenase	406.75	2	811.48	A	DGPLKGIL	G	0.66
54	Glyceraldehyde-3-phosphate dehydrogenase	442.26	2	882.52	A	ADGPLKGIL	G	0.77
55	Glyceraldehyde-3-phosphate dehydrogenase	890.42	1	889.42	V	AVNDPFID	L	0.49
56	Glyceraldehyde-3-phosphate dehydrogenase	530.77	2	1059.52	A	INDPFIDLN	Y	0.60
57	Serum albumin	616.31	2	1230.61	E	FDEKPADLPSL	V	0.53
58	Serum albumin	730.37	2	1458.72	E	FDEKPADLPSLVE	K	0.39
59	Tenascin-R	785.40	1	784.40	M	DNPVDLI	A	0.38
60	Leucine-rich repeat-containing protein	833.40	1	832.52	L	LLLLAPGH	A	0.38
61	Ligoadenylate synthase	473.75	2	945.52	S	EVMIDVLK	V	0.14
62	Fibrous sheath-interacting protein	949.41	1	948.41	G	EAEEFLPD	A	0.21
63	Aspartate aminotransferase	965.52	1	964.52	M	GPPDPILGVT	E	0.83
64	Pyruvate kinase	507.26	2	1012.51	R	GDLGIEIPAE	K	0.18
65	Teashirt homolog	538.27	2	1074.56	V	FDPVVEEKI	Q	0.15
66	Phosphoglycerate mutase	1101.60	1	1100.61	N	LPTGIPIVYE	L	0.19
67	Cas scaffolding protein	601.84	2	1201.69	D	VPTQHRGPVVL	K	0.18
68	Haloacid dehalogenase	636.84	2	1271.69	V	NYILDHLLGSK	–	0.29
69	Glucose-6-phosphate isomerase	641.33	2	1280.66	H	DSSTNGLISFIK	Q	0.32

(*continued on next page*)

Table 1 (*continued*)

No.	Parent Protein	Observed M/Z	Charge	Calculated MW (Da)	P0	Peptide Sequence	Pf	Peptide Ranker Score
70	Myosin	644.35	2	1286.69	M	SNKKPELIDML	L	0.46
71	Complement C3	679.51	2	1356.75	E	VHVLLVNPHTGAT	L	0.18
72	Digestive organ expansion factor	704.78	2	1407.52	E	SEEEDNEEEAEV	E	0.05
73	Ryanodine receptor	491.27	3	1470.79	I	ELGPMTKPLCLKAA	G	0.61
74	Histone	736.90	2	1471.79	R	NDEELNKLLGKVT	I	0.20
75	Neuroblastoma breakpoint family member	742.36	2	1482.62	E	VPEDSQEECAITY	S	0.15
76	Synaptotagmin-like protein	517.26	3	1548.71	L	PFQSSASSPSPSKNE	T	0.30
77	Aggrecan core protein	788.40	2	1574.73	S	LTDTPTLASPEGSGET	E	0.07
78	Cytochrome	509.54	4	2034.13	L	ENPKKYIPGTKMIFAGIK	K	0.69
79	Collagen	1098.54	2	2195.19	G	PPGKPGPPGPPGPPGIQGIHQTL	G	0.55
80	Kielin/chordin-like protein	914.42	3	2740.15	L	PDPLDPTCSLCTCEEGSMRCQKKPC	P	0.80
81	Heat shock protein	966.51	3	2896.51	L	NVLIFDLGGGTFDVSILTIDDGIFEVK	A	0.02
82	Protocadherin	730.36	5	3646.88	P	PATIVPIDEESRNGTILVDNMLIKGTAAGPDPTIE	L	0.02

was analysed using LC-MS/MS and the results were listed in Table 1. A total of 82 peptides were identified with a MW ranging from 439.73 to 3646.88 Da, containing 7 to 35 amino acids. Among these peptides, 64, 62, 43 and 20 peptides contained Pro, Leu, Ala, and Gln, respectively, which had been demonstrated that may facilitate alcohol metabolism *in vivo* (Liang et al., 2014). Moreover, 78 of these peptides contained at least one of the hydrophobic branch-chain amino acids (Val, Leu, and Ile). The ingestion of peptides containing these amino acids, especially short chain peptides, might increase cytoplasmic hydrophobicity and thus activate ADH (Haseba et al., 2006; Xiao et al., 2018). The identified peptides were further screened using the Peptide Ranker program to predict the probability of the peptides to be bioactive, according to their amino acid composition and extracellular status (Mooney, Haslam, Pollastri, & Shields, 2012). Results showed that 25 of the 82 peptides scored higher than 0.5 (Table 1) (Tu et al., 2019).

The potential application of peptides depends not only on bioactivity but also on their bioaccessibility and safety. Potential allergenicity and toxicity are two main concerns for food safety that may hinder peptides application (Gupta et al., 2013; Polikovsky et al., 2019). For instance, although melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) exerted various anti-cancer activities, the non-specific cytotoxicity hindered its applicability in humans (Rady, Siddiqui, Rady, & Mukhtar, 2017). The potential allergenicity and toxicity of these peptides were assessed *in silico* using the AllerTOP and ToxinPred free data analysis tools, respectively. Results are listed in Table S1, together with some physicochemical values of the peptides (i.e., hydrophobicity, amphipathicity, steric hindrance, and pI). The list of 25 peptides with a Peptide Ranker score higher than 0.5 are shown in Table 2. As can be observed, 14 peptides were predicted to be neither allergen nor toxin, while 2 peptides were predicted to exert potential toxicity and 9 peptides allergenicity.

On the other hand, the digestive tract could affect bioactivity and absorption of peptides by modifying their structure (Escudero, Mora, & Toldrá, 2014). GI digestion could also result in the generation of novel potential allergenic, toxic or bioactive peptides (Garcia-Vaquero et al., 2019). As a result, all the 82 identified peptides were subjected to *in silico* digestion.

3.5. In silico digestion, analysis, and screening of the generated peptides

In silico digestion has been considered an economical and time-saving assay in comparison with experimental digestion (Fu et al., 2016). In the present study, all the identified peptides were digested into smaller peptides except SEEEDNEEEAEV. Although SEEEDNEEEAEV seemed to resist GI digestion, it appeared as non-active with a Peptide Ranker score of 0.05. A previous study reported that the most hydrophobic fractions were more sensitive to GI digestion (Xie, Wang, Jiang, Liu, & Li, 2015). In this study, the hydrophobicity of f20 might be the reason of the observed instability of the identified peptides. Meanwhile, a total of 154 peptides were generated after *in silico* digestion, with a molecular weight ranging from 132.14 to 1854.45 Da, containing 2 to 20 amino acids. Likewise, the potential bioactivity, allergenicity, toxicity and physicochemical property of the generated peptides were also accessed (Table S2). Table 3 showed the list of 32 generated peptides with a Peptide Ranker score higher than 0.5. As can be seen, 21 among the 32 peptides were predicted to be neither allergic nor toxic. Results indicated that these peptides with high Peptide Ranker score could be potential ADH stabilizing peptides with applicability.

To further validate the activity of these peptides, 4 among the 21 potential peptides (DPQYPPGPPAF, QKPVL, KPC, and APGH) were chosen for synthesis, and their capacity to stabilize ADH was tested. Main reason that justified the choice of these peptides was that they were obtained after simulated GI digestion, as well as were predicted to be safe and active according to the *in silico* study. Furthermore, these peptides exhibited relatively low steric hindrance and high amphipathicity, which were preferred when ligands binding to the receptor and stabilizing enzyme conformation (Falciani et al., 2007; Manzo et al., 2015).

3.6. ADH stabilizing activity of the synthetic peptides

To validate the effect of synthetic peptides on ADH stability, peptides (5 mM) was mixed with ADH and incubated for 0, 15, 30, 60, 90, and 120 min before the determination of enzyme activity. As can be seen in Fig. 4A, DPQYPPGPPAF, KPC, and APGH stabilized ADH in a similar way than CHs (Fig. 1), whereas QKPVL showed no significant effect on ADH activity. ADH was almost inactivated after 2 h incubation, however, it could retain about 60%, 20%, and 10% of activity with 5 mM KPC, DPQYPPGPPAF, and APGH, respectively. To further understand the dose-effect relationship, different concentrations of peptides (0.01–5 mM) were incubated with ADH for 1 h before determining ADH activity. As shown in Fig. 4B, KPC, DPQYPPGPPAF, and APGH could stabilize ADH in a dose-depend manner, with KPC being the most active. Particularly, KPC could exert significant effect even at a low concentration of 0.01 mM, and it reached the maximum at 5 mM, with ADH activity near to 100%. Interestingly, among the 4 validated peptides, KPC exhibited the highest amphipathicity, while QKPVL showed the highest steric hindrance (Table 3). This might further illustrate that high amphipathicity and low steric hindrance were important for ADH stabilizing activity of peptides. Moreover, it is generally accepted that the bioactivity of a peptide depends on its amino acid composition, the position of amino acid in peptides sequence, and resulting peptide conformation (Sultan et al., 2018; Zheng, Zhao, Dong, Su, & Zhao, 2016). Dipeptide KP had been reported to be an effective enzyme inhibitor (angiotensin converting enzyme and dipeptidyl peptidase IV)

Table 2
Identified peptides with Peptide Ranker score > 0.5 and *in silico* prediction of their allergenicity, toxicity, and physicochemical properties.

No.	Peptide Sequence	Peptide Ranker Score	Allergenicity Prediction	Toxicity Prediction	Steric Hindrance	Amphipathicity	Hydrophobicity	pI	Molecular Weight (Da)
1	DPDDFPL	0.91	non-allergen	Non-Toxin	0.6	0	−0.17	3.43	817.35
2	NKISVVGVGAVGMACAISILMKDLA	0.86	non-allergen	Non-Toxin	0.65	0.29	0.13	8.54	2459.33
3	DPQYPPGPPAFP	0.85	non-allergen	Non-Toxin	0.52	0.1	−0.07	3.8	1281.60
4	ADGPLKGIL	0.77	non-allergen	Non-Toxin	0.6	0.41	0.05	6.19	882.52
5	RDPQYPPGPPAFP	0.76	non-allergen	Non-Toxin	0.53	0.28	−0.2	6.19	1437.70
6	ARDPQYPPGPPAFP	0.72	non-allergen	Non-Toxin	0.53	0.26	−0.16	6.19	1508.74
7	LPVPAFNVINGGSHAGNKL	0.70	non-allergen	Non-Toxin	0.59	0.27	0.03	9.11	1904.03
8	ENPKKYIPGTKMIFAGIK	0.69	non-allergen	Non-Toxin	0.64	0.89	−0.13	9.84	2034.13
9	AGFGGDDAPRAVFP	0.69	non-allergen	Non-Toxin	0.62	0.18	−0.03	4.21	1375.65
10	GFAGDDAPRAVFP	0.67	non-allergen	Non-Toxin	0.61	0.19	−0.04	4.21	1318.63
11	DGPLKGIL	0.66	non-allergen	Non-Toxin	0.62	0.46	0.03	6.19	811.48
12	ATGNPNPDIVWLK	0.60	non-allergen	Non-Toxin	0.6	0.28	−0.06	6.19	1423.75
13	PPGKPGPPGPPGPPGIQGIHQTL	0.55	non-allergen	Non-Toxin	0.51	0.33	−0.03	9.11	2195.19
14	FDEKPADLPSL	0.53	non-allergen	Non-Toxin	0.58	0.45	−0.15	4.03	1230.61
15	PDPLDPTCSLCTCEEGSMRCQKKPC	0.80	non-allergen	Toxin	0.59	0.54	−0.27	4.79	2740.15
16	IPGPPTGPIKF	0.88	non-allergen	Toxin	0.56	0.33	0.08	9.11	1122.64
17	GPPDPILGVT	0.83	allergen	Non-Toxin	0.57	0	0.1	3.8	964.52
18	DDHFLFDKPVSPL	0.75	allergen	Non-Toxin	0.57	0.39	−0.1	4.42	1528.76
19	IDDHFLFDKPVSPL	0.71	allergen	Non-Toxin	0.58	0.37	−0.04	4.42	1641.84
20	GVDNPGHPYIM	0.69	allergen	Non-Toxin	0.59	0.13	0	5.09	1198.54
21	HLDIPKML	0.66	allergen	Non-Toxin	0.54	0.64	−0.03	7.09	965.54
22	ELGPMTKPLCLKAA	0.61	allergen	Non-Toxin	0.57	0.61	−0.04	8.54	1470.79
23	INDPFIDLN	0.60	allergen	Non-Toxin	0.67	0	−0.02	3.57	1059.52
24	VPAFNVINGGSHAGNKL	0.60	allergen	Non-Toxin	0.6	0.3	0.01	9.11	1693.89
25	FPFDVPSEPK	0.58	allergen	Non-Toxin	0.58	0.49	−0.12	4.38	1161.57

(Hatanaka et al., 2012; Ichimura, Hu, Aita, & Maruyama, 2003). This might explain the binding potential of KPC to ADH and enhancing ADH conformation stability. In addition, the Cys residual can contribute to various activities such as antioxidant due to the contribution of the –SH group. From this view, KPC might also protect ADH from oxidative inactivation (Dumont et al., 2018). Results above indicated the ADH stabilizing effect of peptides, although the exact molecular mechanism need further study.

Table 3
Peptides derived from *in silico* GI digestion with Peptide Ranker score > 0.5 and *in silico* prediction of their bioactivity, allergenicity, toxicity, and physicochemical properties.

No.	Peptide Sequence	Peptide Ranker Score	Allergenicity Prediction	Toxicity Prediction	Steric Hindrance	Amphipathicity	Hydrophobicity	pI	Molecular Weight (Da)
1	IF	0.95	non-allergen	Non-Toxin	0.7	0	0.67	5.88	278.37
2	GPPDPIL	0.91	non-allergen	Non-Toxin	0.54	0	0.07	3.8	707.92
3	GG	0.89	non-allergen	Non-Toxin	0.68	0	0.16	5.88	132.14
4	DPQYPPGPPAF	0.88	non-allergen	Non-Toxin	0.53	0.11	−0.07	3.8	1185.44
5	QKPVL	0.82	non-allergen	Non-Toxin	0.59	0.98	−0.16	9.11	583.8
6	KPC	0.71	non-allergen	Non-Toxin	0.55	1.22	−0.38	8.57	346.47
7	PEDEGI	0.70	non-allergen	Non-Toxin	0.64	0.42	−0.19	3.58	658.74
8	APGH	0.65	non-allergen	Non-Toxin	0.39	0.36	−0.02	7.1	380.45
9	GIL	0.61	non-allergen	Non-Toxin	0.64	0	0.47	5.88	301.43
10	AVF	0.59	non-allergen	Non-Toxin	0.64	0	0.47	5.88	335.43
11	PDP	0.57	non-allergen	Non-Toxin	0.49	0	−0.29	3.8	327.36
12	GGDDAPR	0.56	non-allergen	Non-Toxin	0.63	0.35	−0.39	4.21	686.76
13	KPEF	0.56	non-allergen	Non-Toxin	0.6	1.23	−0.3	6.35	519.64
14	ADGP	0.56	non-allergen	Non-Toxin	0.58	0	−0.09	3.8	358.39
15	GDSSVPVW	0.56	non-allergen	Non-Toxin	0.59	0	0.04	3.8	846.01
16	AGR	0.55	non-allergen	Non-Toxin	0.63	0.82	−0.45	10.11	302.36
17	AG	0.55	non-allergen	Non-Toxin	0.6	0	0.21	5.88	146.16
18	PTGIPIV	0.54	non-allergen	Non-Toxin	0.58	0	0.26	5.88	695.96
19	AGDDAPR	0.54	non-allergen	Non-Toxin	0.61	0.35	−0.37	4.21	700.78
20	GGGT	0.53	non-allergen	Non-Toxin	0.64	0	0.07	5.88	290.33
21	DVPGPVL	0.50	non-allergen	Non-Toxin	0.58	0	0.13	3.8	695.91
22	IPGPPTGPIK	0.72	non-allergen	Toxin	0.54	0.37	0.02	9.11	976.33
23	GF	0.99	allergen	Non-Toxin	0.69	0	0.39	5.88	222.26
24	GPM	0.96	allergen	Non-Toxin	0.61	0	0.12	5.88	303.41
25	PSL	0.94	allergen	Non-Toxin	0.47	0	0.07	5.88	315.4
26	NGDK	0.94	allergen	Non-Toxin	0.72	0.92	−0.57	6.19	432.48
27	DPDDF	0.81	allergen	Non-Toxin	0.67	0	−0.32	3.43	607.62
28	VPAF	0.77	allergen	Non-Toxin	0.57	0	0.33	5.88	432.56
29	IM	0.70	allergen	Non-Toxin	0.74	0	0.49	5.88	262.39
30	DGP	0.66	allergen	Non-Toxin	0.6	0	−0.21	3.8	287.3
31	IG	0.50	allergen	Non-Toxin	0.69	0	0.45	5.88	188.25
32	DKPGPPAA	0.50	allergen	Non-Toxin	0.53	0.46	−0.17	6.19	751.93

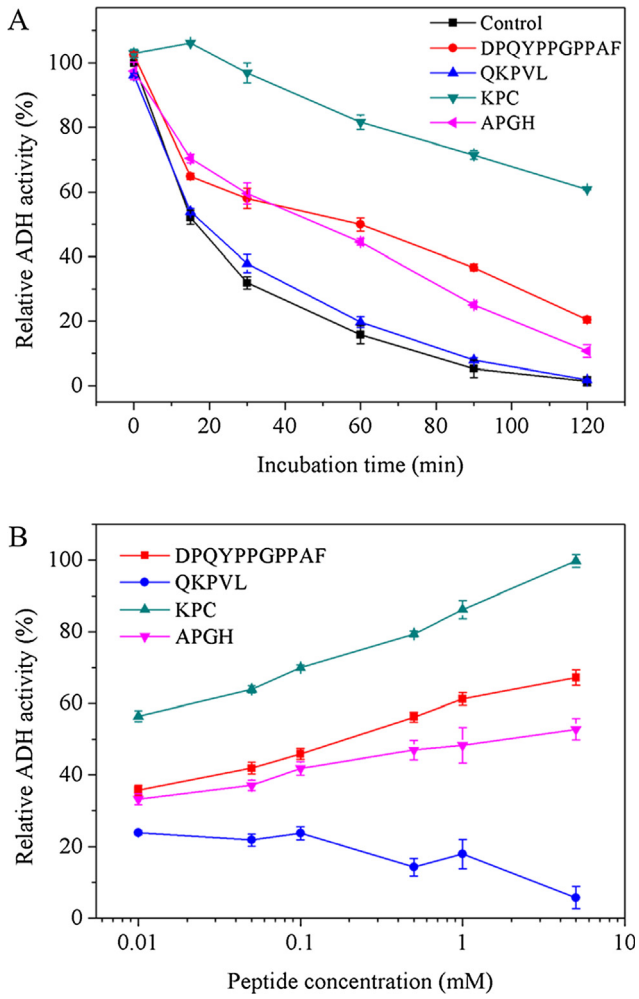

Fig. 4. Effect of synthetic peptides on ADH stability. A: Effect of peptides (0.5 mM) on ADH activity during different incubation times (0–120 min) at 37 °C. B: Effect of different concentration of peptides (0.01–5 mM) on ADH activity (60 min incubation at 37 °C).

4. Conclusion

In this work, we confirmed that CHs could stabilize ADH in a dose-dependent manner and maintain alcohol metabolism at a higher level. A total of 82 peptides were identified from CHs and 154 peptides could be generated from them by *in silico* digestion. Furthermore, 21 among the 154 peptides were predicted to be non-toxic, non-allergic, and possibly active. Three peptides (DPQYPPGPPAF, KPC, and APGH) were synthesized and confirmed as novel ADH stabilizing peptides. Results suggested that chicken hydrolysate could be a good source of bioactive peptides with ADH stabilizing activity and might exert a positive effect on alcoholic liver diseases. Further works would be needed to assess the bioactivity and bioaccessibility of these peptides *in vivo*.

5. Ethics statement

This research did not include any human subjects and animal experiments.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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