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Study of screening, transport pathway, and vasodilation mechanisms on angiotensin-I converting enzyme inhibitory peptide from *Ulva prolifera* proteins

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Abstract

In this study, *Ulva prolifera* protein was used for preparing angiotensin-I converting enzyme (ACE)-inhibitory peptide via virtual gastrointestinal digestion and *in silico* screening. Some parameters of the obtained peptide, such as inhibition kinetics, docking mechanism, stability, transport pathway, were explored by Lineweaver-Burk plots, molecular docking, *in vitro* stimulate gastrointestinal (GI) digestion and Caco-2 cells monolayer model, respectively. Then, a novel anti-ACE peptide LDF (IC_{50}, (1.66 ± 0.34) μmol/L) was screened and synthesized by chemical synthesis. It was a no-competitive inhibitor and its anti-ACE inhibitory effect mainly attributable to four Conventional Hydrogen Bonds and Zn701 interactions. It could keep activity during simulated GI digestion *in vitro* and was transported by peptide transporter PepT1 and passive-mediated mode. Besides, it could activate Endothelial nitric oxide synthase (eNOS) activity to promote the production of NO and reduce Endothelin-1 (ET-1) secretion induced by Angiotensin II (Ang II) in Human Umbilical Vein Endothelial Cells (HUVECs). Meanwhile, it could promote mice splenocytes proliferation in a concentration-dependent manner. Our study indicated that this peptide was a potential ingredient functioning on vasodilation and enhancing immunity.

Key words: *Ulva prolifera* protein, ACE-inhibitory peptide, *in vitro* GI digestion, vasodilation mechanism, immunomodulation

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1 Introduction

Ulva prolifera, an edible green alga, grows on rocks in intertidal zones on mud beaches or on other floating seaweeds on the sea (Tan et al., 1999). This alga contains 9%–14% protein, 2%–3.6% ether extract, 32%–36% ash, and n-3 and n-6 fatty acids 10.4 g/(100 g) and 10.9 g/(100 g) of total fatty acid (Aguilera-Morales et al., 2005). Meanwhile, it's an important source of protein for human consumptions, attributable to its protein composition, containing eighteen kinds of amino acids, including all essential amino acids and a high protein digestibility (98%) (He et al., 2021). The eutrophication of sea water caused by industrial and domestic sewage provides favorable living conditions for *U. prolifera*, resulting in the excess production of this alga (Ye et al., 2011). Nearly all the algae are used as low economical-value food, animal feed, fertilizer or even regarded as industrial waste (Li et al., 2016). Thus, increasing the added value of *U. prolifera* is important in algae's comprehensive utilization.

Hypertension, a chronic disease, afflicting more than 30% of adults worldwide, is the leading cause of cardiovascular disease and premature death (Mills et al., 2020). ACE is a vital enzyme in blood pressure regulation through both the renin-angiotensin system (RAS) and kallikrein kinnin system (KKS), which catalyzes the conversion of inactive Angiotensin I (Ang I) into a potent vasoconstrictor Ang II to inactivate the vasodilator bradykinin (Raghavan and Kristinsson, 2009). Therefore, screening ACE inhibitor is considered as an effective therapeutic approach for the treatment of hypertension. However, some synthetic ACE inhibitors, such as Captopril, Lisinopril, and Enalapril, may lead to significant side effect like inflammatory response, cough, or renal impairment (Ko et al., 2012). Recently, numerous algae proteins have been utilized to isolate ACE inhibitory peptides, such as *Mazzaella japonica* protein (Kumagai et al., 2020), *Gracilariopsis lemaneiformis* protein (Cao et al., 2017) and *Grateloupia asiatica* protein (Sumikawa et al., 2020). However, until now, no anti-ACE peptide purified form *U. prolifera* protein has been synthesized and reached clinical trials.

On the other hand, increasingly, various immunomodulatory peptides have been isolated from plant protein, such as wheat gluten protein (Horiguchi et al., 2005), Juglans regia L protein (Mao et al., 2020), and rice protein (Wen et al., 2021). However, only a few studies of immunomodulatory peptide from *U. prolifera* protein have been reported.

In recent years, *in silico* approaches have been used to predict and screen various anti-ACE peptides released from food-derived proteins, such as rice bran protein (Udenigwe, 2016), Chlorella vulgaris protein (Xie et al., 2018) and rabbit meat protein (Chen et al., 2022). In contrast to bench experiments, it can reduce the time required for screening peptides from different

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protein sources and help to discover new and sustainable precursors of known bioactive peptides (Udenigwe, 2014). Meanwhile, the *in vivo* anti-hypertension effect of peptides is attributable to its stable structure and high absorptivity in the GI tract. Therefore, it is essential to study the stability of bioactive peptides in the GI tract. Simulated GI digestion is a simple, rapid, and inexpensive measure (Lacroix et al., 2017). Differentiated and polarized Caco-2 monolayer is an effective tool to study the transport of peptides *in vitro* due to their morphology, secretion of marker enzymes and permeability characteristics, which are similar to those of the human small intestine (Ding et al., 2021).

Base on the above considerations, we aimed to screen a safe ACE inhibitory peptide from *U. prolifera* protein by *in silico* method. Some pharmacological properties of the selected peptide, such as immunomodulatory activity, GI stability, inhibitory pattern, intestinal tract transport pathway, molecular interactions mechanism of this peptide was also explored. Lastly, the cellular mechanism of the purified peptide on vasodilation was explored on Human Umbilical Vein Endothelial Cells (HUVECs).

2 Materials and methods

2.1 *Materials*

Protein sequences were downloaded from NCBI (https://c1n.cn/wny80) and they were selected for *in silico* analysis. ACE (from rabbit lung), N-hippuril-L-histidy-L-leucine (HHL), Ang II, Human ET-1 ELISA Kit was purchased from Sangon Biotech (Shanghai, China). Nitric Oxide Assay Kit was purchased from Beyotime (Shanghai, China). MTT cell proliferation and cytotoxicity assay kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). HUVECs cell line was purchased from SSRCC (Shanghai, China). All other reagents were of analytical reagent grade.

2.2 *Evaluation of Ulva prolifera proteins as sources of ACE inhibitory peptides*

Fourteen *Ulva prolifera* protein sequences as the precursors of ACE inhibitory peptides were evaluated using the BIOPEP-UWM database (https://c1n.cn/yDaOA). The parameters of proteins, such as name, amino acid number, molecular weight (MW) were shown in Table 1. The frequency (A) of anti-ACE peptides was calculated using the following formula:

$$A = a/N, \tag{1}$$

where a was the number of peptides with anti-ACE activity in a given protein sequence and N was the total number of amino acid residues of protein.

2.3 *In silico digestion and screening*

All the proteins were virtually GI digested by complex enzymes (pepsin and trypsin) in PeptideCutter (expasy.org). Biological activity potential was evaluated at PeptideRanker (ucd.ie). Water solubility was calculated at Innovagen AB (Antibodies, proteins, and peptides). Toxicity was explored at ToxinPred (osdd.net). Human intestinal absorption (HIA) was tested at admetSAR (ecust.edu.cn). Finally, the affinities of these peptides against the ACE were evaluated by molecular docking in Discovery Studio 2020 software (DS 2020, Accelrys, San Diego, CA, USA).

2.4 *Peptide synthesis and activity validation*

The selected biopeptides were synthesized by China Peptides Co., Ltd. (Suzhou, China) and the purity of the peptide was ⩾98%. The ACE inhibitory rate was explored according to previous reported (Pan et al., 2016). The IC_{50} value was defined as the inhibitor concentration inhibiting 50% activity of ACE.

2.5 *Inhibition kinetics of the synthetic peptide.*

The inhibition pattern of the synthetic peptides was explored by Lineweaver-Burk plots. The concentrations of the ACE substate (HHL) were set as 1 mol/L, 2.5 mol/L and 5 mol/L. Peptide concentrations were set as 0 μmol/L, 10 μmol/L and 100 μmol/L. V_{max} and K_m were measured as the y-axis and x-axis intercepts of the primary plot, respectively.

2.6 *Stability of the synthetic peptides against GI proteases*

Peptides solution was adjusted to pH 2.0 (using 1 mol/L HCl). Pepsin (1% E/S (enzyme/substrate), w/w) was added to the solution and the mixture was incubated at 37℃ for 2 h. Then, half of the juice was terminated by boiling water and the other half was adjusted to pH 7.5, and followed digested by trypsin (1% E/S, w/w) for another 2 h at 37℃. Then, the reaction was also terminated by boiling water and all the peptide solutions were adjusted to pH 7.0. Subsequently, both solvents were centrifuged at 12 000 r/min for 5 min. The supernatants were analyzed using Q-Exactive mass spectrometer. Besides, the ACE inhibitory activity of the processed peptide solutions was determined at the same concentration (5 μmol/L).

Table 1. *Ulva prolifera* protein sequences used in *in silico* analysis

Protein	Amino acid residues	Molecular weight/kDa	A
Pyruvate orthophosphate dikinase	899	96.43	0.162 4
Adenine phosphoribosyl transferase	182	19.19	0.390 8
Photosystem Ⅰ assembly protein Ycf4	185	24.42	0.451 0
Ribosomal protein L14 (chloroplast)	123	13.52	0.401 6
50S ribosomal protein L5 (chloroplast)	179	20.22	0.372 2
30S ribosomal protein S12 (chloroplast)	74	13.60	0.365 9
γ-carbonic anhydrase 1	173	23.57	0.381 5
γ-carbonic anhydrase 2	226	17.99	0.327 4
Plastid geranylgeranyl diphosphate synthase	330	35.69	0.248 5
Plastid isopentenyl-diphosphate delta-isomerase Ⅰ	245	27.66	0.326 5
Plastid 4-cytidine-5-diphospho-2-C-methyl-D-erythritol kinase	324	35.45	0.250 0
Plastid 4-diphosphocytidyl-2C-methyl-D-erythritol synthase	269	29.24	0.304 8
Plastid 1-deoxy-D-xylulose 5-phosphate reductoisomerase*	437	46.94	0.251 7
Plastid 1-deoxy-D-xylulose 5-phosphate synthase	713	76.88	0.186 5

Note: Parameter A: the frequency of bioactive fragments occurring in a protein sequence. *, represents the precursor protein for the final selected peptide.

2.7 *Molecular docking*

ACE crystal structure (ID: 1O8A) was optimized in DS 2020, removing water molecules and ligand, adding hydrogen and then was set as a receptor. The three-dimensional structure of the selected peptide was constructed, followed and carried for energy minimization optimization. Then, followed a docking program between the selected peptide and ACE special binding sites (coordinates *x*, 40.79; *y*, 33.61; and *z*, 43.38) was carried by C docking. Lisinopril was selected as a control candidate. The other parameters were set as default values except for special instructions.

2.8 *Proliferative effects of peptides on splenocyte*

Preparation of mice splenocytes refers to our previous report (Li et al., 2020). Splenocyte proliferation assay was conducted by MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) and the cell viability rate was calculated as follows:

$$\begin{aligned}\text{Cell viability}\ (\%) =&(\text{Optical density of test well}-\\&\text{optical density of spontaneous})\times\\&100/(\text{Optical density of control}-\\&\text{Optical density of spontaneous}).\end{aligned}\quad(2)$$

2.9 *Effect of the selected peptide on HUVECs*

HUVECs were cultured in Ham's F-12K (0.1 mg/mL Heparin, 0.03–0.05 mg/mL ECGs, 10% FBS, 100 U/mL 1% penicillin and 100 U/mL streptomycin) at 37℃ and 5% CO_2. Peptides with gradient concentrations (0 μmol/L, 25 μmol/L, 50 μmol/L and 100 μmol/L) were used to culture HUVECs.

Intracellular NO content determination: 100 μL of this cell (5×10^5 cells/mL) was seeded in 96-well plates and cultured by peptides for 12 h, before adding 3-amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate (DAF-FM DA) (20 μmol/L) for another 30 min. Then, the intracellular fluorescence intensity of HUVECs was explored by Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan). The excitation and emission wavelength were 495 nm and 515 nm, respectively.

Extracellular NO content determination: HUVECs were cultured by peptides in the same condition as above and the NO content in medium was explored according to Nitric Oxide Synthase Assay Kit (Beyotime, Shanghai, China). The extracellular UV optical density was explored by microplate reader (Varioskan LUX, Thermo Scientific, USA) at 540 nm.

Intracellular Endothelial nitric oxide synthase (eNOS) activity determination: HUVECs were cultured by peptides in the same condition as above and the eNOS activity was explored according to Nitric oxide synthase test kit (Beyotime, Shanghai, China).

ET-1 secretion determination: HUVECs were cultured in 96-well plates for 12 h with the same condition as above, before adding Ang II (100 nmol/L, final concentration) for another 6 h. Finally, the content of ET-1 in processed cells was determined according to Human ET-1 ELISA Kit (Sangon Biotech, Shanghai, China).

2.10 *Statistical analysis*

Data were analyzed by one-way analysis of variance (ANOVA), and then Dunnett multiple tests was performed using GraphPad Prism Version 9 (San Diego, CA, USA). Values (mean ± SD) that do not share a common superscript lowercase letter within a column differ significantly ($p < 0.05$) ($n = 3$).

3 Results and discussion

3.1 *Screening of ACE inhibitory peptides*

A previous study has confirmed that some peptide fragments present in *U. prolifera* protein could inhibit the activity of ACE (Pan et al., 2016). Thus, fourteen proteins from the algae were used as precursors to produce a novel anti-ACE peptide by *in silico* analysis (Table 1). Parameter *A* represents the frequency of anti-ACE peptides present in a given protein sequence. Various proteins with a given *A* value higher than 0.3 have been published, such as quinoa proteins (0.345 1 to 0.420 8) (Guo et al., 2020), rabbit meat protein (0.327 5 to 0.866 6) (Chen et al., 2022) and yak milk casein (0.441 2 to 0.655 5) (Lin et al., 2018). Therefore, to sum up, the higher values of parameter *A*, the more potential anti-ACE peptides in a given protein sequence (Iwaniak et al., 2020). In this study, the *A* value of the precursor protein was 0.251 7, lower than that of the above-mentioned studies. Thus, we speculated that the high *A* value was not the determining factor in producing anti-ACE peptides.

In recent years, virtual GI digestion as cheap and efficient technology to produce peptide, has been more and more prominent in anti-ACE peptides production (Chen et al., 2022). Some pharmacological properties, such as the biological activity potential, toxicity, solubility, absorptivity and penetration, should be considered in the screening of novel anti-ACE peptides (Ferreira and Andricopulo, 2019). In addition, small peptides usually have higher anti-ACE activity (Pan et al., 2016). Therefore, peptides smaller than pentapeptide were collected and a total of 654 peptides were obtained after *in silico* proteolysis (Table S1). Among of them, twelve peptides (Peptide ranker >0.8) exhibited better ADMET characteristics (Table 2). However, among of these peptides, only LDF could dock with ACE. Subsequently, this peptide was synthesized and acquired an IC_{50} of (1.66 ± 0.34) μmol/L *in vitro* (Fig. 1). Compared to the selected LDF, various othered reported anti-ACE peptides with similar sequence, such as LDY (IC_{50}, 6.10 μmol/L) (Furuta et al., 2016), DFG (IC_{50}, 44.7 μmol/L) (Balti et al., 2010), CDF (IC_{50}, 192.17 μmol/L) (Chen et al., 2022), VDF (IC_{50}, 6.59 μmol/L) and LPF (IC_{50}, 10.59 μmol/L) (Majumder and Wu, 2009). Thus, we speculated that L (Leu) in N-terminal residues or F (Phe, aromatic amino acids) in C-terminal residues may play a key role in the anti-ACE activity of peptides. It was similar to the previous report that aromatic or cyclic amino acids (F, Phe) were the most common C-terminal residues of highly active ACE inhibitors (Xie et al., 2018).

Table 2. Pool of potential ACE inhibitory peptides

Peptide	Peptide ranker	WS	Toxin	HIA	BBB	-CE score
LDF	0.839 471	Good	NO	+0.759 1	+0.895 2	75.045 9
WKL	0.827 822	Good	NO	+0.649 3	+0.855 6	Fail
FLK	0.805 333	Good	NO	+0.759 1	+0.837 1	Fail
FLKF	0.956 004	Good	NO	+0.759 1	+0.837 1	Fail
FLPR	0.925 723	Good	NO	+0.655 4	+0.806 6	Fail
DLGW	0.876 197	Good	NO	+0.649 3	+0.910 9	Fail
LSRF	0.817 020	Good	NO	+0.713 9	+0.959 5	Fail
LDLF	0.806 570	Good	NO	+0.759 1	+0.895 2	Fail
RYIF	0.847 108	Good	NO	+0.751 9	+0.940 3	Fail
DFL	0.889 906	Good	NO	+0.759 1	+0.895 2	Fail
LDFL	0.831 537	Good	NO	+0.759 1	+0.895 2	Fail
LYRF	0.920 545	Good	NO	+0.751 9	+0.918 6	Fail

Note: WS, water solubility; HIA+, high human intestinal absorptivity; BBB +, higher blood brain barrier permeability; -CE score, score of -C Docker energy (–kcal/mol).

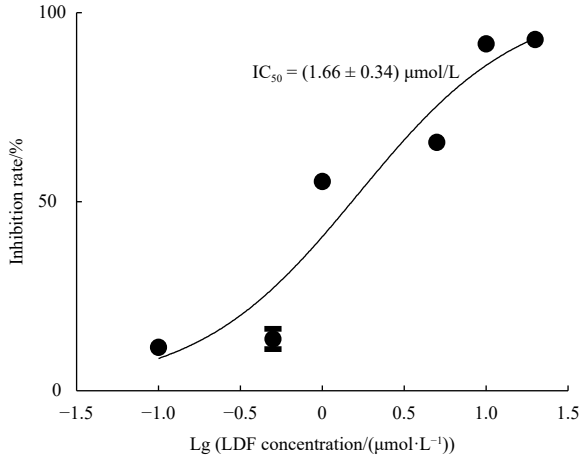

Fig. 1. IC_{50} value determination of the selected peptide.

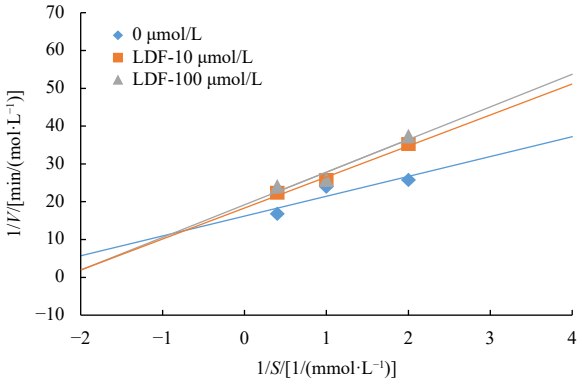

Fig. 2. Lineweaver-Burk plots of the synthetic peptide. $1/V$ and $1/S$ represents the reciprocal of reaction velocity and substrate concentration, respectively.

3.2 *Inhibitory kinetics of peptides on ACE*

Anti-ACE peptides were reported to play three patterns, including competitive, non-competitive, and mixed-competitive to bind with ACE (Maeno et al., 1996; Xie et al., 2018). In this study, as peptide concentration increased (0–100 μmol/L), Km remained constant while Vmax decreased, which was indicative of non-competitive inhibition (Fig. 2). Similarly, for instance, the peptide CDF from rabbit meat protein was a non-competitive inhibitor. Both of them interacted with free protease and/or protease-substrate complex with similar affinity to inhibit the activity of ACE (Sangsawad et al., 2018).

3.3 *Stability of peptide against pepsin, pepsin-trypsin.*

In order to determine the stability of the synthetic peptides during the incubation of GI proteases, a simulated GI digestion study was performed *in vitro*. As shown in Fig. 3, some of the peptides were degraded by pepsin and no noteworthy changes were found after incubation with trypsin. Fortunately, there was no significant change in ACE inhibitory activity of the processed

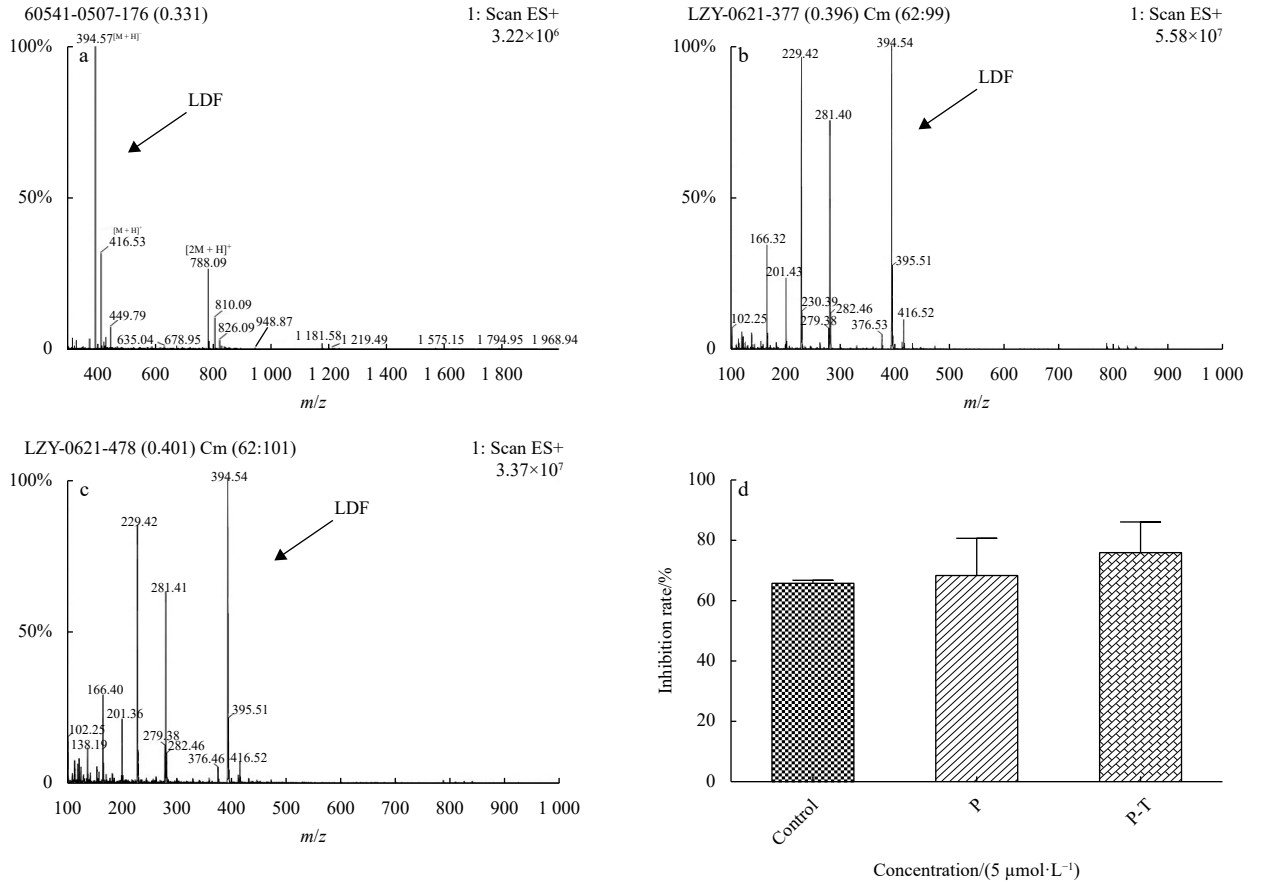

Fig. 3. Stability of LDF against GI proteases. Full scan primary MS chromatogram for Control group (a), pepsin (b), pepsin-trypsin (c). d presents inhibition rate of above groups in the same concentration (P, pepsin; P-T, pepsin-trypsin; 5 μmol/L). The values are represented as the mean of the triplicate ± SD.

peptides (Fig. 3d). Anti-ACE peptides processed by GI proteases showed different effects: an increase in the activity of the WMY peptide, no change in the IY peptide, and a decrease or even loss in the activity of other synthesized peptides (Xu et al., 2021). It was probable that the small peptide fragments from the GI digestion process have an inhibitory function on ACE.

3.4 *Transepithelial transport of peptides across the Caco-2 cell monolayer*

Caco-2 cell monolayers transport system is an effective model to study the absorption of bioactive peptides (Hidalgo et al., 1989). The basal surface (B side) of Caco-2 cell model is equivalent to the inner wall of the intestine and has an efflux function. In this study, the result indicated that transport rate of peptide LDF exhibited a time-dependent relationship and up to a maximum value of 6.47% at 90 min (Fig. 4a). Similarly, the transport time was consistent with the anti-ACE peptide LVLPGE (Pei et al., 2021). To determine which pathways were involved in the peptide transport, the effects of different modulators were investigated. Wortmannin, a transcytosis inhibitor, had no effect on the transport of LDF, suggesting that endocytosis was not the major transport mode. Gly-Pro was a suitable substrate for peptide transporter of PepT1 and could combine with the majority of PepT1 in the intestinal model. Compared to the control group, we found that the Papp of Gly-Pro treated group was decreased significantly ($P < 0.05$) (Fig. 4b). Therefore, we speculated that LDF was transported by PepT1. Meanwhile, Cytochalasin D was a disruptor of tight junction (TJ), and could significantly increase the transport of LDF ($P < 0.05$), suggesting that TJ was involved in the transport process (Fig. 4b). Therefore, the passive paracellular transport via TJ was involved in the transport process of LDF. A large number of water-soluble short peptides were transported by paracellular pathway, such as LVLPGE (Pei et al., 2021), KYIPIQ (Lin et al., 2020), IQW and LKP (Xu et al., 2017). In this study, LDF was consistent with the above reported peptides. Meanwhile, the monolayers of Caco-2 cells were tighter than that of human intestinal epithelial cells, which was not conducive to peptide transport (Conradi et al., 1993). In contrast, human intestine contains more carrier-mediated transports and a larger effective absorbing area (Ding et al., 2021). Therefore, the transport of LDF *in vivo* may be superior to that of the Caco-2 cells model.

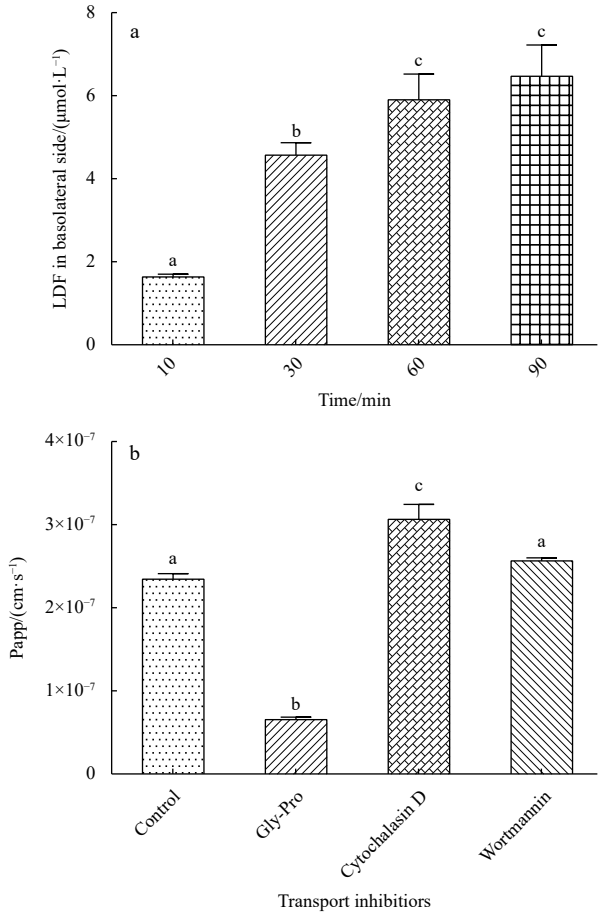

Fig. 4. Effect of incubation time on the transport of LDF across the Caco-2 cell monolayers. a. Transport rate of LDF at different times. Samples were collected from the B side at 30 min, 60 min, and 90 min for HPLC analysis. Effect of Gly-Pro (PepT1 inhibitor), cytochalasin D (Tight junction disruptor) and wortmannin (Transcytosis inhibitor) on the transport of LDF across Caco-2 cell monolayers.Values represent the mean±standard deviation, and the bars with different lowercase letters were significantly different ($p < 0.05$, $n = 3$).

3.5 *Molecular docking*

Molecular docking is a key tool in exploring the binding mechanism of inhibitor and ACE (Chen et al., 2022). As shown in Table 3, there were four types of bonds between LDF and ACE, including Electrostatic interaction, Conventional Hydrogen Bond, Carbon Hydrogen Bond, and Alkyl. The most stabilized pose of LDF was mainly attributable to the four conditional Hydrogen bonds, which docked onto the key sites, such as HIS353 in S1 pocket and TYR520 in S2 pocket, respectively. Meanwhile, some key sites in S2 pocket, such as HIS353, HIS513, and LYS511 were linked by Electrostatic interaction and Carbon Hydrogen Bond, respectively. In contrast, there were four types of bonds between Lisinopril and ACE, including Pi-Cation, Attractive Charge, Carbon Hydrogen Bond, Conventional Hydrogen Bond. Notably, the Conventional Hydrogen Bond number of Lisinopril was higher than LDF, which may contribute to the lower IC_{50} of Lisinopril (IC_{50} = 1.1 ng/mL) *in vitro* (Fig. 5). In addition, zinc is an important catalytic component of ACE (Natesh et al., 2003). Paradoxically, LDF as a non-competitive inhibitor can bind with the active site (Zn701) of ACE. Pervious study indicated that some non-competitive anti-ACE peptides, such as PFPGPIPN (Lin et al., 2017), TTW and VHW (Xie et al., 2018) can also bind with the active site of ACE during the molecular docking process. We speculated that the dock result is related to the evaluation mechanism of docking software, in which all the potential dock poses of peptide with ACE will be exhibited.

3.6 *Effect of peptide on mice splenocytes.*

A large body of evidence shows that bioactive peptides regulate immunity by enhancing lymphocyte proliferation and antibody synthesis (Yang et al., 2009). As shown in Fig. 6, the proliferative effect of LDF on mice splenocytes exhibited concentration-dependence. Some peptides with pro-proliferative activity usually contain a high proportion of hydrophobic amino acids, such as FFSDL (Sütas et al., 1996), FLPFNQL (Liu et al., 2020), RGPPP (Yang et al., 2020) and NGLAP (Hou et al., 2012). Thus, the hydrophobic amino acids Leu (L) and Phe (F) may play a key role in the proliferative effects of LDF. Meanwhile, immune cells play a major role in blood pressure control and have a critical impact as a therapeutic target for hypertension (Liu et al., 2017). The *in vivo* immunity and blood pressure regulation by LDF in mice need to

Table 3. Interactions between ACE and candidates

Candidates	Bond position	Distance/Å	Type	Number
LDF	A:LYS511:HZ3-LDF:O54	1.816 86	Electrostatic interaction	5
	A:ASP415:OD2-LDF:H4	2.087 41	–	–
	A:HIS353:NE2-LDF:O31	5.149 2	–	–
	A:HIS353:NE2-LDF:O54	4.022 29	–	–
	A:ZN701:ZN-LDF:O31	2.316 03	–	–
	A:HIS353:HE2-LDF:O33	2.683 86	Conventional Hydrogen Bond	4
	A:TYR520:HH-LDF:O53	2.287	–	–
	A:ASP415:OD1-LDF:H2	2.525 46	–	–
	A:ASP415:OD1-LDF:H3	2.544 77	–	–
	A:HIS353:HE1-LDF:O33	2.625 87	Carbon Hydrogen Bond	4
	A:HIS353:HE1-LDF:O54	2.320 17	–	–
	A:VAL380:HA-LDF:O21	2.323 24	–	–
	A:HIS513:HE1-LDF:O54	2.348 5	–	–
	A:VAL380-LDF:C16	5.395 25	Alkyl	1
Lisinopril (Lis)	A:HIS353:NE2-Lis:O1	5.546 54	Attractive Charge	3
	A:LYS511:NZ-Lis:O22	5.338 06	–	–
	A:ZN701:ZN-Lis:O1	2.067 3	–	–
	A:GLN281:HE21-Lis:O22	2.452 66	Conventional Hydrogen Bond	6
	A:GLN281:HE22-Lis:O23	2.282 18	–	–
	A:HIS353:HE2-Lis:O17	2.465 11	–	–
	A:TYR520:HH-Lis:O23	2.028 26	–	–
	A:ALA354:O-Lis:H40	2.119	–	–
	A:HIS383:NE2-Lis:H58	2.011 19	–	–
	A:HIS353:HE1-Lis:O17	2.546 83	Carbon Hydrogen Bond	3
	A:GLU162:OE2-Lis:H52	2.778 82	–	–
	A:GLU384:OE1-Lis:H59	2.295 22	–	–
	A:HIS383-Lis:N24	4.959 65	Pi-Cation	1

Note: – reprents no data.

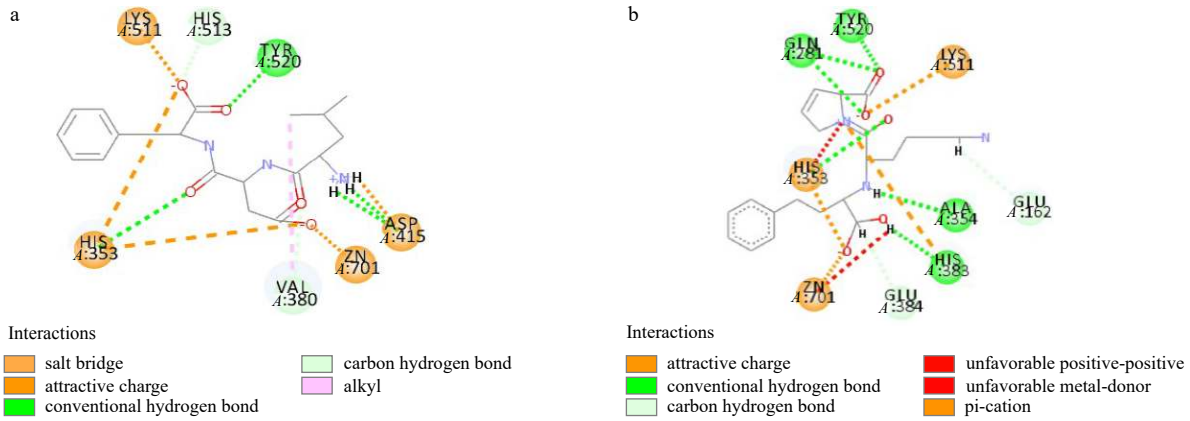

Fig. 5. Docking simulation of candidates binding with ACE, a-LDF (IC_{50} = 1.66 μmol/L), b-Lisinopril (ID:1O86, IC_{50} = 1.1 ng/mL).

be further studied.

3.7 *Effect of this peptide on eNOS activity, NO and EF-1 secretion in HUVECs*

HUVECs line the internal surface of blood vessels, and represent a good model to evaluate the molecular mechanisms involved in blood pressure regulation (García-Tejedor et al., 2015). In this study, *In vitro* HUVECs incubation was performed to explore the vasodilation mechanism of LDF. As shown in Fig. 7a, LDF (100 μmol/L) could promote the secretion of NO significantly ($p < 0.05$) by activating the activity of eNOS ($P < 0.05$). Similarly, the green fluorescence value indicated that, the intracellular NO content increased significantly under the effect of peptides (Fig. 7c). Bradykinin, a peptide fragment containing nine amino acids, acted to promote the production of NO by endothelial cells and it could be degraded by ACE (Fukuda et al., 2009). Hence, we speculated that the anti-ACE effect of LDF reduced the breakdown of bradykinin to promote the activation of eNOS and NO generation. In HUVECs, eNOS converts the L-arginine into L-citrulline and Endothelial NO and it is tightly regu-

lated by co- and post-translational lipid modifications, and phosphorylation on multiple residues (Robb et al., 2004). eNOS-derived NO, an endogenous vasodilatory gas, functioned on vasorelaxation via the activation of vascular smooth muscle soluble guanylate cyclase (sGC) and maintained an anti-proliferative and anti-apoptotic environment in the vessel's wall (Zabel et al., 1998; Sessa, 2004). However, which signaling pathway play vital role in mediating LDF-stimulate eNOS activation and NO production needs to be further studied. ET-1, an endothelium-derived peptide, was one of the most potent vasoconstrictors (Miyauchi et al., 1990). Ang II could promote the secretion of ET-1 in a concentration-dependent manner though the upregulation of the ET-1 mRNA and its effects were prevented by specific protease inhibitors (Imai et al., 1992). Pervious study indicated vasoconstriction to ET-1 and thromboxane is augmented in the face of eNOS deficiency (Lamping and Faraci, 2003). Similarly, LDF could induce eNOS activation and prevent the Ang II-induced overexpression of ET-1 ($P < 0.05$) (Fig. 7b). These results suggested the potential anti-hypertension effect of LDF *in vivo*. In addition, ET-1 could induce NAD(P)H oxidase subunit gp91phox mRNA expression to enhance superoxide anions generation in HUVECs (Duerrschmidt et al., 2000). Therefore, LDF could alleviate vascular endothelial oxidative damage mediated by ET-1.

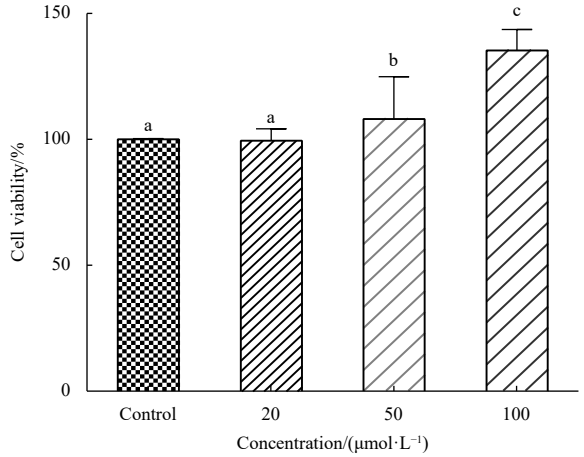

Fig. 6. Effect of peptide on the proliferation of mice splenocytes.

4 Conclusions

In this study, a novel anti-ACE peptide LDF (IC_{50}, (1.66 ±

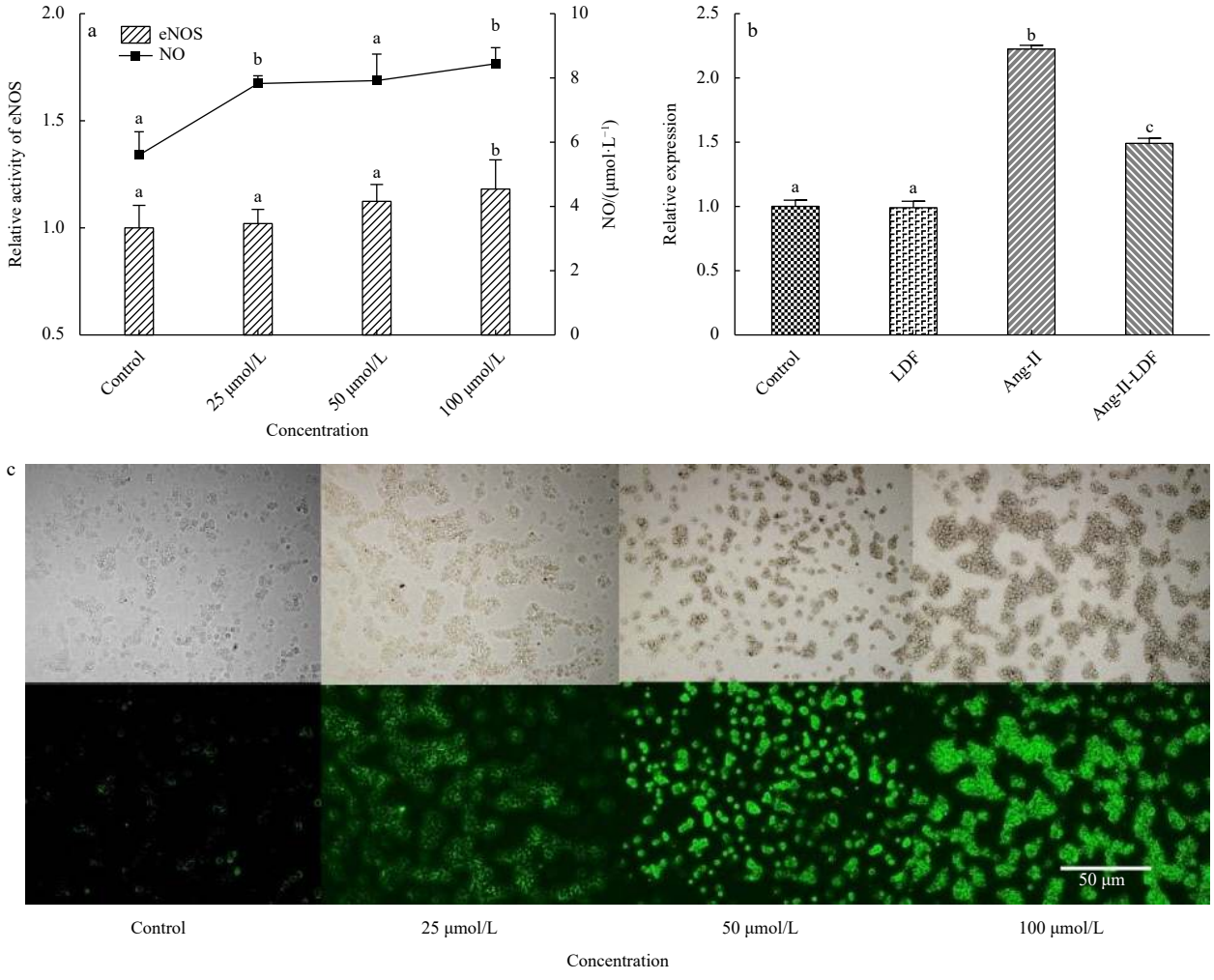

Fig. 7. Effect of LDF on HUVECs. a. Effect of LDF on eNOS activity and NO secretion (extracellular). b. Effect of LDF on ET-1 secretion (Control, cells were cultured by medium for 18 h; LDF, cells were cultured by LDF for 18 h; Ang II, cells were cultured by medium for 12 h and then, adding Ang-II for another 6 h; Ang-II-LDF, cells were incubated by LDF for 12 h and then, adding Ang II for another 6 h; The final concentration of LDF and Ang II were 100 μmol/L and 100 nmol/L, respectively). c. Effect of LDF on the NO secretion (intracellular). Values (mean ± SD) that do not share a common lowercase letter within a column differ significantly ($p < 0.05$) ($n = 3$).

0.34) μmol/L) was screened from *Ulva prolifera* protein. It was a no-competitive inhibitor and its anti-ACE inhibitory effect mainly was attributable to four Conventional Hydrogen Bonds and Zn701 interactions. It could keep activity after simulated GI digestion *in vitro* and was transported by peptide transporter PepT1 and passive-mediated mode through Caco-2 cells monolayers. Besides, in HUVECs, LDF could activate eNOS activity to promote the production of NO and reduce ET-1 secretion induced by Ang II. In addition, LDF could promote mice splenocytes proliferation in a concentration-dependent manner. Our study indicated that LDF was a potential drug ingredient to relax blood vessels and enhance immunity.

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Supplementary information:

Table S1. Peptides produced by *in vitro* stimulate gastrointestinal digestion.

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