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Interactions of *C. frondosa*-derived inhibitory peptides against angiotensin I-converting enzyme (ACE), α -amylase and lipase

Yi Zhang, Shudong He, Xin Rui, Benjamin Simpson

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24 **Abstract**

25 The study illustrates the molecular mechanisms by which marine-derived peptides
26 exhibited different structures and inhibition functions to concurrently inhibit multiple enzymes
27 involved in chronic diseases. Peptides (2 mg/mL) exhibited inhibition against angiotensin-
28 converting enzyme (ACE, inhibition of 52.2–78.8%), pancreatic α -amylase (16.3–27.2%) and
29 lipase (5.3–17.0%). Further *in silico* analyses on physiochemistry, bioactivity, safety and
30 interaction energy with target enzymes indicated that one peptide could inhibit multiple enzymes.
31 Peptide FENLLEELK potent in inhibiting both ACE and α -amylase showed different
32 mechanisms: it had ordered extended structure in ACE active pocket with conventional H-bond
33 towards Arg₅₂₂ which is the ligand for activator Cl⁻, while the peptide folded into compact “lariat”
34 conformation within α -amylase active site and the K residue in peptide formed intensive H-
35 bonds and electrostatic interactions with catalytic triad Asp₁₉₇–Asp₃₀₀–Glu₂₃₃. Another peptide
36 APFPLR showed different poses in inhibiting ACE, α -amylase and lipase, and it formed direct
37 interactions to lipase catalytic residues Phe₇₇ & His₂₆₃.

38

39 **Keywords**

40 Peptides; inhibition; ACE; α -amylase; lipase

41

42 **1. Introduction**

43 Peptides produced from foods can perform inhibitory activities against enzymes involved
44 in chronic diseases. Although synthetic inhibitor compounds are mainly used to combat chronic
45 diseases, their negative side effects such as hepatotoxicity, necessitate the discovery of natural
46 bioactive peptides as alternatives (Daliri, Lee, & Oh, 2018). Angiotensin I-converting enzyme
47 (ACE, EC 3.4.15.1) plays a key role in the regulation of blood pressure via renin-angiotensin
48 system and kallikrein-kinin system by means of converting inactive decapeptide angiotensin-I
49 into potent vasoconstrictor angiotensin-II, as well as inactivating the vasodilator bradykinin
50 (Toopcham, Mes, Wichers, Roytrakul, & Yongsawatdigul, 2017). Pancreatic α -amylase (EC
51 3.2.1.1) catalyzes the hydrolysis of α -linked polysaccharides (e.g., starch) for glucose production,
52 and its inhibition results in a reduction of blood glucose levels after carbohydrate diet for
53 controlling type 2 diabetes (Admassu, Gasmalla, Yang, & Zhao, 2018). Lipase (EC 3.1.1.3) acts
54 specifically on the ester bonds of triglycerides to release free fatty acids, mono- and di- glyceride,
55 and its inhibitors, e.g., a commercialized drug “orlistat”, can delay the absorption of free fatty
56 acids into systemic circulation and adipocytes (Rajan, Palaniswamy, & Mohankumar, 2020) for
57 obesity management. Moreover, hypertension, diabetes and obesity are correlated, and unhealthy
58 diet is one of their fundamental causes. Thus, it is of great interest to discover and understand the
59 mechanisms of food-derived peptides with inhibitory activity against enzymes such as these.

60 The source of the peptides in this research is a marine species, Atlantic sea cucumber
61 (*Cucumaria frondosa*). Our previous study disclosed antioxidant peptides from *C. frondosa* via
62 enzymatic hydrolysis (Zhang, He, Bonneil, & Simpson, 2020b) similar to antioxidant peptides
63 from foods such as rice bran and beans (Ngoh & Gan, 2016; Wang, Chen, Fu, Li, & Wei, 2017),
64 and these peptides with multi-functions have potential research and application value. Currently,

65 *in silico* approaches based on peptide database and computational programs are widely used in
66 combination with conventional *in vitro/in vivo* methods to study bioactive peptides in terms of
67 screening, proteolysis, bioactivity, toxicity and allergenicity, and to derive information on their
68 binding conformations and interactions with enzymes (Kang, Jin, Lee, Kim, Koh, & Lee, 2020;
69 Zhang, Aryee, & Simpson, 2020a). In this study, peptides with inhibitory activities against ACE,
70 pancreatic α -amylase, and pancreatic lipase were obtained *in vitro*, and the identified peptide
71 sequences were analyzed using *in silico* tools to assess their properties and interpret inhibition
72 mechanisms.

73

74 **2. Materials and methods**

75 **2.1. Enzymes, chemicals, and peptides**

76 ACE (from rabbit lung), α -amylase (from porcine pancreas), lipase (Type II, from porcine
77 pancreas), and chemicals including acetonitrile (ACN), 3,5-dinitrosalicylic acid (DNS), ethyl
78 acetate, formic acid, Hip-His-Leu, maltose, olive oil, phenolphthalein, starch, and triton X-100
79 were purchased from Sigma-Aldrich Co., USA. Peptides were produced from fresh *C. frondosa*
80 using alcalase (A) and trypsin (T) with concentrations of 3000 and 2000 U/g at a ratio of 1:3
81 (w/v), respectively. The reaction mixture was incubated with agitation at 25 °C for 2 h;
82 afterwards, the mixture was heated at 100 °C for 1 min to inactivate enzymes, and further filtered
83 with 0.22 μ m membranes (Millipore, USA). Peptides were fractioned using 10 kDa, 5 kDa, 3
84 kDa and 2 kDa cut-off ultrafiltration membranes (Millipore, USA) centrifuged at 3000 g, and 14
85 peptide fractions ($A_{<10}$, A_{10-5} , $A_{<5}$, A_{5-3} , $A_{<3}$, A_{3-2} , $A_{<2}$, and $T_{<10}$, T_{10-5} , $T_{<5}$, T_{5-3} , $T_{<3}$, T_{3-2} , $T_{<2}$)
86 were obtained. The peptides in $A_{<2}$ and $T_{<2}$ fractions were subsequently selected based on their
87 relatively high multi-enzymes inhibitory properties and identified using LC-MS/MS and Peaks

88 software (Zhang et al., 2020b).

89 **2.2. Determinations of multi-enzyme inhibitory activities**

90 ACE inhibitory activity of peptides was determined based on the method described
91 previously (Zhang, Li, Fu, & Mou, 2019) with Hip-His-Leu as substrate to produce hippuric acid
92 at 37 °C, pH 8.3, and the reaction was terminated by HCl and measured spectrophotometrically
93 at 280 nm. Alpha-amylase inhibitory activity of the peptides was determined using starch as
94 substrate to produce maltose at 37 °C, pH 6.0, and the reaction was terminated by adding the
95 color reagent containing DNS, potassium sodium tartrate and NaOH then measured
96 spectrophotometrically at 540 nm (Cardullo et al., 2020). The amount of generated maltose was
97 calculated using a standard curve (0–0.2%, w/v). Lipase inhibitory activity of peptides was
98 determined using olive oil as substrate to produce free fatty acids at 37 °C, pH 7.5. The assay
99 was terminated by boiling the reaction mixture before measuring fatty acids by titration with
100 NaOH using phenolphthalein as indicator (Huang, Chow, & Tsai, 2012).

101 **2.3. Characterizations of peptide biochemical properties**

102 Peptide sequences were submitted to various *in silico* tools listed in **Table S1**.
103 Physiochemical properties including isoelectric point (pI), water solubility and hydrophobicity of
104 the peptides were predicted using Peptide property calculator. Probability of bioactivity was
105 predicted using PeptideRanker (Han, Maycock, Murray, & Boesch, 2019). Inhibitory activities
106 towards ACE, α -amylase and lipase were predicted by BIOPEP-UWM using “Bioactive peptides”
107 database and “Analysis” program. Location of bioactive fragments in a peptide and the
108 frequency of bioactive fragments occurrence (parameter A) were returned (Minkiewicz, Iwaniak,
109 & Darewicz, 2019). Toxicity was predicted using ToxinPred where SVM-based method with
110 threshold value of 0.0 was selected (Gupta et al., 2013). Allergenicity was predicted using

111 “Allergenic proteins with their epitopes” database in BIOPEP-UWM.

112 **2.4. Molecular docking**

113 Molecular docking between each peptide and enzymes ACE, α -amylase and lipase,
114 respectively, was performed using BIOVIA Discovery Studio (Accelrys, San Diego, CA, USA).
115 The crystal structures of human ACE (PDB ID: 1O8A), human pancreatic α -amylase (PDB ID:
116 5KEZ) and human pancreatic lipase (PDB ID: 1LPB) were obtained from Protein Data Bank
117 (PDB). The enzyme structures were optimized to minimize the energy and to remove water
118 molecules before docking. CDOCKER docking program was applied where a more negative
119 CDOCKER energy, i.e., higher (-)CDOCKER energy value, indicated more favorable docking
120 affinity, and “F” means docking failure (Zhang et al., 2020b). The binding site of each enzyme
121 contained its active site, and the values of binding site were adjusted according to those reported
122 for enzyme – reference peptide interaction as follows: binding sites for ACE were created using
123 the coordinates x: 36.189, y: 43.643, z: 55.175 and a radius of 16 Å. A known ACE inhibitory
124 peptide, PRY (Fu, Alashi, Young, Therkildsen, & Aluko, 2017) was used as control (C1). The
125 docking of α -amylase was carried out with a radius of 16 Å and coordinates x: -5.95348, y:
126 3.30228, z: -25.4401. A known α -amylase peptide, YPYSCWVRH (Goldbach et al., 2019) was
127 used as control (C2). For lipase, binding site with a radius of 15 Å and coordinates x: 8.02469, y:
128 23.5388, z: 55.7064 was created. A known lipase inhibitory peptide, YFS (Stefanucci et al., 2019)
129 was used as control (C3).

130 **2.5. Statistical analyses**

131 Triplicate measurements were performed for enzyme inhibition determinations. Statistical
132 significance was analyzed using SPSS 22.0 (IL, USA).

133

134 3. Results and discussion

135 3.1. *In vitro* multi-enzyme inhibitory activities of peptides

136 As shown in **Fig. 1**, the peptides strongly inhibited ACE (52.2–78.8%) followed by α -
137 amylase (16.3–27.2%), and least for pancreatic lipase (5.3–17.0%). It suggests that *C. frondosa*
138 could be source material to produce enzyme-inhibitory peptides. Other closely related species
139 recently reported with ACE inhibitory peptide activities include *Stichopus japonicus* and
140 *Holothuria atra* (Dewi, Patantis, Fawzya, Irianto, & Sa'diah, 2020; Zhong, Sun, Yan, Lin, Liu,
141 & Cao, 2018). However, peptides derived from sea cucumber with α -amylase and lipase
142 inhibitory activities have not been reported.

143 ACE inhibitory activity (**Fig. 1a**) showed that the peptides with relatively low MW, i.e., <2
144 kDa and 2-3 kDa, had significantly high ACE inhibitory activity ($p < 0.05$) which agreed with
145 previous findings on *Acaudina molpadioidea* peptides (Zhao, Li, Liu, Dong, Zhao, & Zeng,
146 2007). Peptide fractions A_{<2} and T_{<2} (MW <2 kDa, 2 mg/mL) from *C. frondosa* inhibited ACE
147 activity by 78.8% and 65.4%, respectively, which were higher than their counterparts generated
148 from *H. atra* (57.4%, MW <3 kDa, 1 mg/mL) (Dewi et al., 2020) and *S. japonicus* gonads (50%,
149 MW <3 kDa, 1.32 mg/mL) (Zhong et al., 2018). Previously found peptides exhibiting high α -
150 amylase inhibitory activity were mainly from plant sources (Awosika & Aluko, 2019), and only
151 a few from aquatic sources, e.g., shrimp and red seaweed (Admassu et al., 2018; Yuan, Li, Pan,
152 Wang, & Chen, 2018). This study indicated that sea cucumber is a potential marine source of α -
153 amylase inhibitory peptides. Peptide fractions A_{<2} and T_{<2} had 27.2% and 24.1% α -amylase
154 inhibitory activities, respectively (**Fig. 1b**). Collagen is the main protein component in sea
155 cucumber, and a study on camel skin collagen hydrolysates also found peptides with potent ACE
156 and pancreatic α -amylase inhibitory activities (Mudgil, Jobe, Kamal, Alameri, Al Ahabbi, &

157 Maqsood, 2019). Inhibition effect of the peptides on pancreatic lipase was low (**Fig. 1c**) with A_{<2}
158 and T_{<2} showing 18.3% and 15.3% inhibitory activities against lipase, respectively. Similarly,
159 peptides produced from yellow field pea showed both α -amylase and lipase inhibitory activities,
160 and 50% reduction in lipase activity was achieved by a high concentration (4–12 mg/mL) of
161 peptides (Awosika et al., 2019).

162 **3.2. *In silico* analyses of peptide properties**

163 Peptide sequences obtained from *de novo* sequencing with both average local confident
164 (ALC) values $\geq 90\%$ and local confidence (LC) values $\geq 70\%$ were selected (**Table 1**). There
165 were 11 sequences in A_{<2}, and 32 sequences in T_{<2}. All peptides consisted of 5–9 amino acids.
166 Peptides in A_{<2} had pI values from 5.10 to 11.29, and 46% were water soluble, while peptides in
167 T_{<2} had broader pI values from 3.85 to 11.39, and 72% showed high water solubility. Potential
168 anti-health properties were predicted that no peptide was toxic and allergenic, suggesting the
169 peptides had low possibility to pose a food safety issue.

170 The potential bioactivity of each peptide was assessed (**Table 1**). Peptide YDWRF (No. 1
171 in A_{<2}) had the highest possibility to be the most bioactive, and peptide WDLFR (No. 4 in A_{<2},
172 No. 30 in T_{<2}) had the 2nd highest score. In total, 55% peptides in A_{<2} had bioactive potential
173 ≥ 0.8 , but only 9% peptides in T_{<2} fraction had bioactivity ≥ 0.8 , suggesting that alcalase produced
174 more bioactive peptides than trypsin. Specifically, 100% of peptides in A_{<2} fraction and 87.5%
175 of peptides in T_{<2} fraction were found with ACE inhibitory fragments. The composition of
176 certain amino acids, including aromatic, hydrophobic, basic, and positively-charged amino acids
177 contribute to ACE inhibitory activity (Lee & Hur, 2017). In the study, peptide EVPLFR with
178 parameter A value of 1.0 was found with ACE inhibitory fragments EV, VP, PL, LF, LFR and
179 FR that comprised of aliphatic amino acids (V, L), aromatic amino acids (F, R, P), and they

180 provided high affinity towards the active site of ACE due to hydrophobicity (Lee et al., 2017; Li,
181 Le, Shi, & Shrestha, 2004). No α -amylase and lipase inhibitory fragments were found in peptides,
182 which was due to the lack of relevant dataset in BIOPEP-UWM. Admassu et al. (2018) reported
183 2 α -amylase inhibitory peptides, GGSK and ELS, produced from red seaweed. Ngoh et al. (2016)
184 reported 7 α -amylase inhibitory peptides from Pinto beans that possessed amino acids such as L,
185 P, G and F, similar to the data from this work. Findings on lipase inhibitory peptides are still
186 insufficient. ϵ -Polylysine (25–30 K residues) is commercially used as food additives in Japan
187 because of its inhibition against human and porcine pancreatic lipase. Tripeptides containing
188 high content of R residues were synthesized and exhibited strong inhibition against lipase
189 (Stefanucci et al., 2019). Peptides NPVWKRK and CANPHELPNK derived from *Spirulina*
190 *platensis* decreased the accumulation of triglyceride by binding pancreatic lipase (Fan, Cui,
191 Zhang, & Zhang, 2018). In this study, several peptides containing these characteristic amino
192 acids that were responsible for the inhibition of α -amylase and lipase were observed (**Fig. 1**).

193 **3.3 Inhibition mechanisms of peptides inhibiting multi-enzymes**

194 According to the (-)CDOCKER energy data (**Table 1**) that present the overall energy
195 consumed by peptide as inhibitor when bound with enzyme active site, all the produced peptides
196 possessed inhibitory activities against ACE and pancreatic α -amylase. Six peptides were able to
197 inhibit ACE, pancreatic α -amylase and lipase. Peptides generated by trypsin generally exhibited
198 higher inhibitory activities than alcalase produced peptides, which differed with the results
199 obtained using peptide database in subsection 3.2. For each peptide, the data from molecular
200 docking only matched those returned from online tools to a small extent. It suggested that the
201 structure of the peptides played a key role in inhibiting enzymes. The control peptide C1, PRY,
202 is a strong competitive inhibitor of ACE by forming H-bond (Fu et al., 2017) and it showed a

203 docking energy of -57.7345 . All peptides produced from *C. frondosa* had lower docking energy
204 values, indicating they were stronger ACE inhibitors than PRY. The control peptide C2,
205 YPYSCWVRH, is a selective and competitive inhibitor against α -amylase (Goldbach et al.,
206 2019), and its binding to pancreatic α -amylase consumed energy of -134.391 . Peptides
207 FENLLEELK, FNTDSALER, SYNELLTK in this study appeared to be stronger α -amylase
208 inhibitors than C2 as they consumed much lower energies. The control tripeptide C3, YFS,
209 showed a CDOCKER energy of -61.3976 against pancreatic lipase. Among the 6 peptides
210 successfully docked onto lipase, peptide APFPLR had a similar docking energy value to C3, and
211 the other 5 peptides appeared to be stronger inhibitors than C3. The peptides that failed in
212 docking with lipase were too large to fit in lipase active site. The structure–function relationship
213 of multi-enzyme inhibitory peptide was illustrated by peptide FENLLEELK (**Fig. S1a**), which
214 had robust inhibitory activities against ACE and pancreatic α -amylase, as well as peptide
215 APFPLR (**Fig. S1b**) which showed moderate inhibitory activities against ACE, pancreatic α -
216 amylase and lipase.

217 3.3.1. Inhibition mechanisms of peptide FENLLEELK

218 The overall structure of human ACE is in an ellipsoid shape with a central groove that
219 extends to the internal enzyme molecule, and peptide FENLLEELK buried in the groove (**Fig.**
220 **2a**). It had an extended conformation that enabled its access to ACE active site crevice (**Fig. 2b**);
221 thus, was able to prevent zinc, the essential cofactor of ACE, from binding with the conserved
222 HEXXH zinc binding motif in ACE (His₃₈₃, His₃₈₇, Glu₄₁₁) (Natesh, Schwager, Sturrock, &
223 Acharya, 2003) located at the bottom of peptide–ACE interaction surface. Each residue in the
224 peptide participated in the interaction with atoms in ACE active site (**Fig. 2c**). The F residue on
225 the N-terminal of the peptide formed desirable carbon hydrogen bonds with Asn₆₆ (2.45 \AA),

226 Try₆₂ (2.45 Å) but the interaction was slightly weakened by unfavorable positive-positive
227 interaction with Arg₁₂₄ (4.67 Å) (Zhao, Zhang, Yu, Ding, & Liu, 2020). The K residue on the
228 peptide C-terminal formed attractive charge interaction with Asp₁₂₁ (4.35 Å) and a salt bridge
229 with Glu₁₂₃ (1.9 Å) via lysyl side chain, apart from attractive charge interaction with Arg₁₂₄ (5.38
230 Å) and a conventional H-bond with Trp₂₂₀ (2.87 Å) from the –COO⁻ group. FENLLEELK had 3
231 E residues and 3 L residues. The carboxyl side chain from E residues formed strong electrostatic
232 interactions with Arg₁₂₄, His₄₁₀, Arg₅₂₂, Lys₁₁₈, as well as a conventional H-bond with Arg₅₂₂
233 which was reported as the chloride ion ligand for ACE activation for substrate hydrolysis
234 (Natesh et al., 2003). The isobutyl group of L residues formed hydrophobic interactions with
235 Trp₅₉, Phe₃₉₁ and Val₅₁₈. The interactions between peptide and ACE active site including H-bond,
236 electrostatic and hydrophobic effects were intense.

237 Peptide FENLLEELK was also buried in the active site cleft of human pancreatic α -
238 amylase as seen from the top view of the surface (**Fig. 3a**). Compared to the pose when docking
239 with ACE (**Fig. 2b**), the same peptide formed a more compact pose in a unique lariat fold when
240 interacting with lipase (**Fig. 3b**). Previous research found that lariat-type peptides were potent
241 and highly selective to α -amylase over other glycosidases (Jongkees, Caner, Tysoe, Brayer,
242 Withers, & Suga, 2017). The six residues on peptide N-terminal, i.e., FENLLE formed a head-to-
243 side chain macrocycle, while the C-terminal residue K stretched to the base of the active site
244 cleft to form interactions with atoms in α -amylase active pocket. The macrocycle composed of
245 six amino acids was stabilized by various intermolecular H-bond and charge interactions (**Fig. 3c**)
246 and was responsible for a few interactions with the α -amylase active site. The F residue formed
247 pi-charge electrostatic bond with Trp₅₉; the N residue formed conventional H-bond with Val₃₅₄
248 (2.61 Å); the L residue formed electrostatic interactions with Val₃₅₄ (4.34 Å) and His₃₀₅ (4.88 Å);

249 however, the E residue formed an undesirable charge repulsion with negatively charged Asp₃₅₆
250 (5.3 Å) (**Fig. 3d**). The “tail” of the lariat peptide residue K towards the end of active site pocket,
251 was the one contributing the most to the inhibition, due to the large number of interactions to the
252 active atoms especially the catalytic triad in α -amylase (Asp₁₉₇–Asp₃₀₀–Glu₂₃₃). Although it
253 formed an unfavorable donor-donor clash with Ala₁₉₈, the –NH₃ group on the side chain made
254 two conventional H-bonds (2.38 Å, 2.7 Å) and one attractive charge interaction (4.33 Å) with
255 Asp₁₉₇, the catalytic nucleophile of α -amylase, and one electrostatic interaction (4.75 Å) with
256 Asp₃₀₀, as well as one conventional H-bond (1.96 Å) and one salt bridge (2.07 Å) with Glu₂₃₃
257 which is the catalytic acid-base of α -amylase (Goldbach et al., 2019).

258 3.3.2. Inhibition mechanisms of peptide APFPLR

259 The pose of APFPLR in ACE active site was extended to block the entrance for substrate
260 binding. The binding between peptide and non-catalytic residues in ACE active site was
261 facilitated by electrostatic interactions, hydrophobic interactions and H-bonds (**Fig. S2a**).
262 Conversely, peptide APFPLR engaged intensively with pancreatic α -amylase. It formed a curled
263 structure among the A, P, F, P residues and stretched its R residue to the bottom of the α -amylase
264 active site with its guanidino group in the side chain interacting with the α -amylase catalytic triad
265 with both H-bonds and attractive charge interactions (**Fig. S2b**).

266 Peptide APFPLR was one of the several peptides that docked on human pancreatic lipase, a
267 receptor used in this study in an activated form and in complex with co-lipase, an amphiphilic
268 protein facilitating the opening of the lid domain (**Fig. 4a**) to allow efficient lipolysis at a hostile
269 lipid-water interface (Stefanucci et al., 2019). APFPLR formed a stretched pose with its C-
270 terminal towards the end of the interaction surface to occupy the narrow and shallow active site
271 groove of lipase (**Fig. 4b**), and its catalytic triad Ser₁₅₂–Asp₁₇₆–His₂₆₃ was blocked at the bottom

272 of the interaction surface of lipase–peptide complex (**Fig. 4c**). A strong interaction network was
273 formed between the peptide and the residues in lipase active pocket (**Fig. 4d**), and the comprised
274 residues A, P and L are aliphatic that favored the binding with lipase active site. The A residue
275 on peptide N-terminal formed H-bonds with Thr₂₁ and Glu₂₂. Other residues in the peptide
276 mainly made hydrophobic interactions with lipase active site atoms. For example, the pyrrolidine
277 side chain of P residue made a hydrophobic interaction towards Cys₁₈₁; the benzyl side chain of
278 F residue made hydrophobic interactions with Ile₂₀₉ and Val₂₁₀; and the L residue formed
279 hydrophobic interactions with Phe₇₇, Pro₁₈₀, Tyr₁₁₄, Ala₁₇₈, among which the Phe₇₇ is the H-bond
280 donor and electrostatic stabilizer to form oxyanion hole for substrate hydrolysis (Eydoux et al.,
281 2008). The R residue on C-terminal interacted with Asp₇₉ and Phe₇₇ with conventional H-bond
282 and/or charge-charge interaction via its –NH₃ group. Moreover, the C-terminal (–COO[–]) made a
283 carbon H-bond (2.4 Å) and a charge-charge interaction (4.82 Å) with His₂₆₃, which is in the
284 catalytic triad to be attacked by Asp₁₇₆ and acts as a general base in lipase catalysis (Eydoux et
285 al., 2008).

286

287 **4. Conclusions**

288 Peptides derived from *C. frondosa* inhibited ACE, α -amylase and lipase based on *in vitro*
289 and *in silico* data. Peptide with the same sequence adjusted its conformational structure to bind
290 towards the active sites of multi-enzymes. FENLLEELK exhibited potent inhibitory activities
291 against ACE and α -amylase by forming different poses and weak interactions. APFPLR inhibited
292 pancreatic lipase due to the amphipathicity of the constituent amino acids, structural features and
293 intense interactions with catalytic residues. This research illustrated the molecular mechanisms
294 and structure–function relationship of multi-enzyme inhibitory peptides against different

295 enzymes.

296

297 **Declaration of Competing Interest**

298 The authors declare no conflict of interest.

299

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303

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Table 1

The sequences, physiochemical properties, bioactivities, safety and docking energies of inhibitory peptides.

No.	Peptide sequence	De novo sequencing		Physicochemical property		Bioactivity (Peptide Ranker)	Enzyme inhibitory activity			Safety		(-)CDOCKER Energy		
		ALC (%)	LC (%)	pI	H ₂ O solubility		ACE	α -amylase	Toxicity	Allergenicity	ACE	α -amylase	lipase	
														Location of fragments
Alcalase-produced peptides														
1	YDWRF	97	96 97 98 98 97	6.36	Good	0.965565	YDWRF	0.2000	---	No	---	112.639	101.845	F
2	VVLLPLR	95	95 96 100 100 88 94 93	10.8	Poor	0.375063	VVLLPLR	0.4286	---	No	---	112.618	100.133	F
3	LDLPLR	95	92 90 97 97 98 95	6.64	Good	0.601238	LDLPLR	0.5000	---	No	---	107.664	117.235	F
4	WDLFR	94	94 86 98 97 94	6.52	Good	0.956441	WDLFR	0.6000	---	No	---	88.8559	81.5657	F
5	ELPPHFL	93	90 88 96 97 95 93 96	5.10	Poor	0.821660	ELPPHFL	0.4286	---	No	---	108.682	84.4674	74.5224
6	APFPLR	92	84 88 93 93 98 95	10.9	Poor	0.947738	APFPLR	0.8333	---	No	---	81.5545	70.4191	60.7478
7	VPFLPR	92	90 87 96 97 92 90	10.8	Poor	0.797496	VPFLPR	0.3333	---	No	---	80.8358	75.3007	F
8	PLQLRP	91	96 99 96 94 81 82	11.3	Good	0.599230	PLQLRP	0.8333	---	No	---	85.5900	76.1232	F
9	TEFHLL	91	90 96 73 91 97 97	5.10	Poor	0.395475	TEFHLL	0.5000	---	No	---	129.975	104.348	F
10	PVFPLR	90	96 97 97 74 86 92	11.3	Poor	0.864694	PVFPLR	0.6667	---	No	---	86.2038	68.7906	F
11	EVPLRFW	90	88 79 94 98 88 90 93	6.86	Good	0.830047	EVPLRFW	0.7143	---	No	---	112.161	95.4367	F
Trypsin-produced peptides														
1	VELWR	97	93 100 98 98 97	6.84	Good	0.298764	VELWR	0.4000	---	No	---	107.935	105.588	F
2	WALLVDAPR	96	90 99 100 99 96 96 97 98 97	6.52	Poor	0.419358	WALLVDAPR	0.4444	---	No	---	141.922	126.433	F
3	LLVNFR	96	98 99 96 92 96 97	10.8	Poor	0.429748	LLVNFR	0.3333	---	No	---	121.291	116.337	F
4	VVNLWR	96	98 97 94 94 96 97	10.8	Poor	0.378438	VVNLWR	0.1667	---	No	---	114.319	110.854	F
5	QAPVKPR	96	95 98 98 95 96 96 95	11.4	Good	0.422813	QAPVKPR	0.7143	---	No	---	104.272	97.6040	F
6	LQLWR	96	94 94 97 96 99	10.8	Good	0.704684	LQLWR	0.4000	---	No	---	103.409	108.075	F
7	FPSLVGR	95	96 97 99 100 98 95 82	10.6	Poor	0.643490	FPSLVGR	0.4286	---	No	---	105.061	99.8073	F
8	LEEQSR	95	94 98 98 88 95 98	4.15	Good	0.0511313	LEEQSR	0.1667	---	No	---	148.099	120.492	F

9	WLDVLR	95	96 95 93 93 97 96	6.52	Good	0.631447	<u>WLDVLR</u>	0.3333	---	No	---	123.579	121.832	F
10	LLMELR	95	97 96 88 96 97 97	6.86	Good	0.345431	<u>LLMELR</u>	0.3333	---	No	---	131.147	121.354	F
11	LLLEMEK	94	79 97 97 98 95 99 98	4.15	Good	0.0988306	<u>LLLEMEK</u>	0.2857	---	No	---	159.526	131.090	F
12	LLELLK	94	89 89 98 96 99 97	6.85	Good	0.157782	<u>LLELLK</u>	---	---	No	---	135.126	117.700	F
13	FENLLEELK	93	79 93 95 91 96 98 98 98 98	3.85	Good	0.163453	<u>FENLLEELK</u>	0.1111	---	No	---	205.065	167.002	F
14	MNLPFR	93	86 93 96 93 96 97	10.6	Poor	0.902172	<u>MNLPFR</u>	0.3333	---	No	---	110.060	105.355	F
15	FVLELR	93	82 92 96 98 98 96	6.62	Good	0.319959	<u>FVLELR</u>	0.1667	---	No	---	135.643	120.277	F
16	EYVFR	93	92 90 93 96 97	6.81	Good	0.351391	<u>EYVFR</u>	0.8000	---	No	---	113.542	119.242	F
17	LNLDLLR	93	92 91 94 90 92 96 96	6.64	Good	0.457278	<u>LNLDLLR</u>	0.2857	---	No	---	144.478	131.423	93.0810
18	SNNVLR	93	85 85 97 94 98 98 96	10.6	Poor	0.673915	<u>SNNVLR</u>	0.4286	---	No	---	136.041	118.587	F
19	LENVLR	93	91 96 86 90 97 98	6.86	Good	0.126053	<u>LENVLR</u>	0.1667	---	No	---	137.731	124.212	F
20	VMQDQVLR	93	90 95 91 91 89 93 99 97	6.61	Good	0.222451	<u>VMQDQVLR</u>	0.1250	---	No	---	159.909	137.249	F
21	KVSWR	92	84 95 92 93 97	11.4	Good	0.330550	<u>KVSWR</u>	---	---	No	---	107.854	112.654	F
22	SYNELTK	92	84 86 93 95 86 97 99 97	6.58	Good	0.213744	<u>SYNELTK</u>	0.2500	---	No	---	169.344	148.307	F
23	LLQLAR	92	95 97 86 91 90 92	10.8	Poor	0.252639	<u>LLQLAR</u>	0.5000	---	No	---	108.805	109.644	F
24	FNTDSALER	92	82 82 97 96 88 89 98 100 96	3.93	Good	0.191898	<u>FNTDSALER</u>	---	---	No	---	174.249	159.707	F
25	LNFEPR	91	91 87 92 97 89 95	6.86	Good	0.470901	<u>LNFEPR</u>	0.8333	---	No	---	129.363	101.454	72.3177
26	TTDVLR	91	85 82 93 94 98 96	6.33	Good	0.0895658	<u>TTDVLR</u>	0.1667	---	No	---	124.179	119.971	94.1367
27	FEQFFK	91	87 96 97 98 95 72	6.61	Good	0.833484	<u>FEQFFK</u>	---	---	No	---	148.119	122.455	F
28	FVTVLR	90	89 89 86 88 96 96	10.6	Poor	0.259446	<u>FVTVLR</u>	0.1667	---	No	---	116.674	113.835	F
29	MANLQR	90	88 92 90 92 88 95	10.6	Poor	0.257394	<u>MANLQR</u>	0.1667	---	No	---	128.732	114.027	82.5811
30	WDLFR	90	82 85 96 94 95	6.52	Good	0.956441	<u>WDLFR</u>	0.6000	---	No	---	112.303	115.031	F
31	ELFDPR	90	92 86 89 91 89 93	3.93	Good	0.561531	<u>ELFDPR</u>	0.3333	---	No	---	127.572	98.7788	F
32	EVPLFR	90	92 85 92 88 87 96	6.86	Good	0.535712	<u>EVPLFR</u>	1.0000	---	No	---	113.853	98.6151	F

Notes: In the “Enzyme inhibitory activity” category, the location of fragments with ACE inhibitory bioactivity were presented with blue underlines for each peptide. The “---” means no data returned from the online tools. The “F” in the “(-)CDOCKER Energy” category means the failure of molecular docking of the peptide with human pancreatic lipase.

Figure Captions

Fig. 1. ACE inhibitory activity **(a)**, pancreatic α -amylase inhibitory activity **(b)**, and pancreatic lipase inhibitory activity **(c)**, of peptides produced via alcalase (A) or trypsin (T) hydrolysis (MW ranges of <10 kDa, 10–5 kDa, <5 kDa, 5–3 kDa, <3 kDa, 3–2 kDa and <2 kDa). Different letters in alcalase (A)-produced peptides (a, b, c, d, e) or trypsin (T)-produced peptides (a', b', c') means significant difference ($p < 0.05$). Significance levels of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were for A- versus T-produced peptides.

Fig. 2. Molecular interaction between inhibitory peptide FENLLEELK and human ACE. Top view of FENLLEELK binding with ACE **(a)**. Molecular surface (H-bond donor and H-bond acceptor in pink and green, respectively) between peptide and ACE active site (peptide molecule in stick and colored by element; key residues of ACE zinc-binding motif in yellow stick) **(b)**. Interactions of ACE–peptide complex (interacting residues of ACE shown in different color based on different interactions) **(c)**.

Fig. 3. Molecular interaction between inhibitory peptide FENLLEELK and human pancreatic α -amylase. Top view of FENLLEELK binding with α -amylase **(a)**. Molecular surface (H-bond donor and H-bond acceptor in pink and green, respectively) between peptide and α -amylase active site (peptide molecule in stick and colored by element; key residues of α -amylase in yellow stick) **(b)**. FENLLEELK in lariat shape **(c)**. Interactions of α -amylase–peptide complex (interacting residues of α -amylase shown in different color based on different interactions) **(d)**.

Fig. 4. Molecular interaction between inhibitory peptide APFPLR and human pancreatic lipase. Pancreatic lipase–co-lipase–peptide complex **(a)**. Top view of APFPLR binding with lipase **(b)**. Molecular surface (H-bond donor and H-bond acceptor in pink and green, respectively) between peptide and lipase active site (peptide molecule in stick and colored by element; key residues of lipase in yellow stick) **(c)**. Interactions of lipase–peptide complex (interacting residues of lipase shown in different color based on different interactions) **(d)**.

Fig. 1

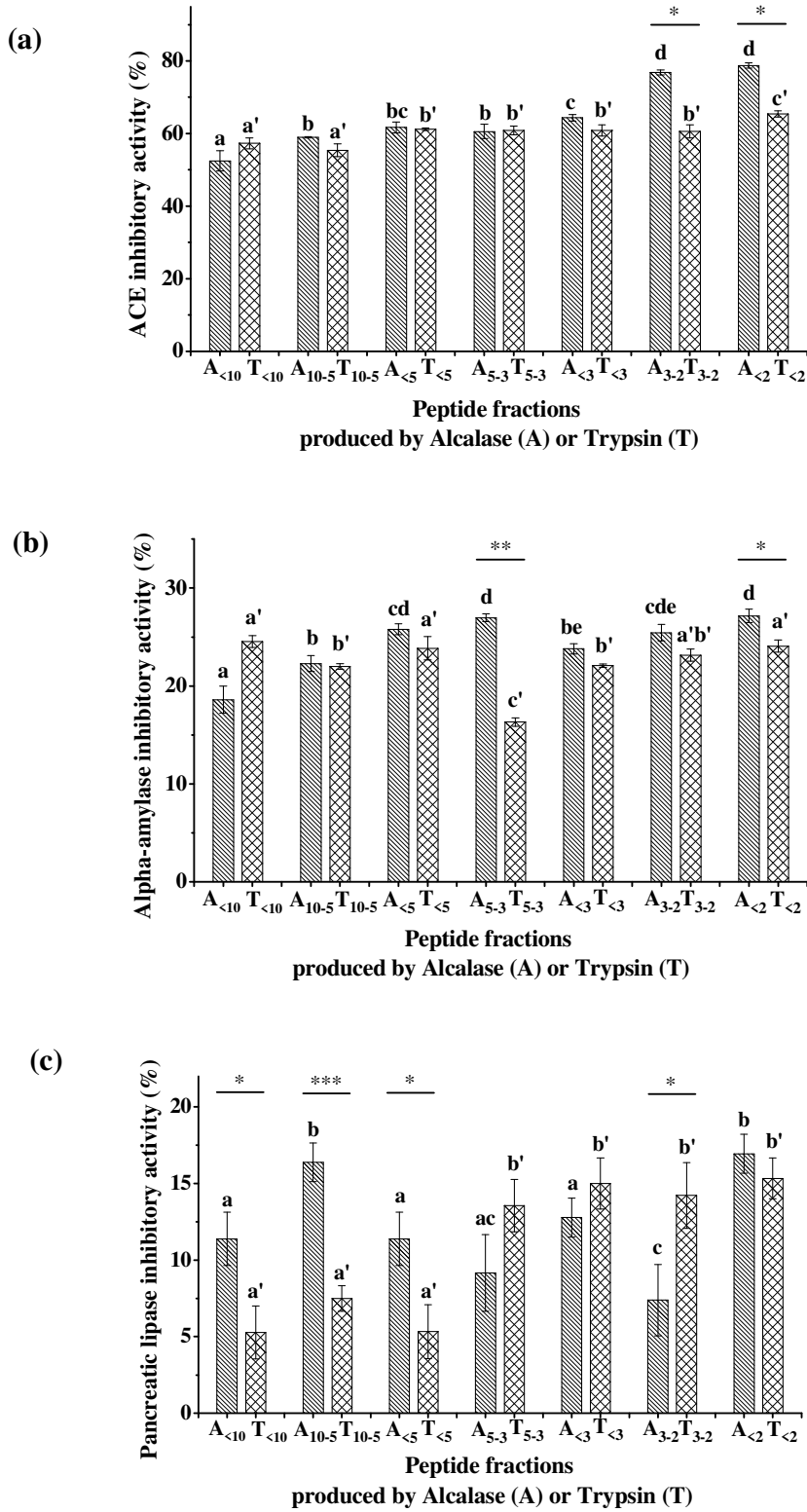
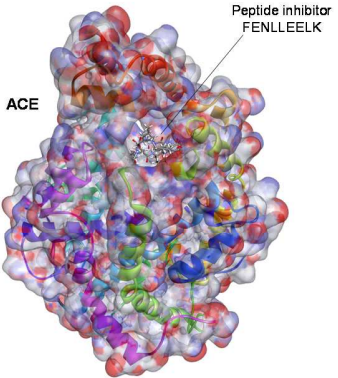
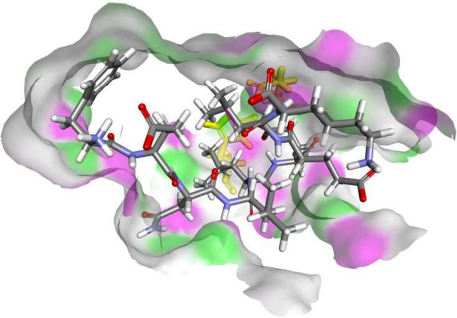


Fig. 2

(a)



(b)



(c)

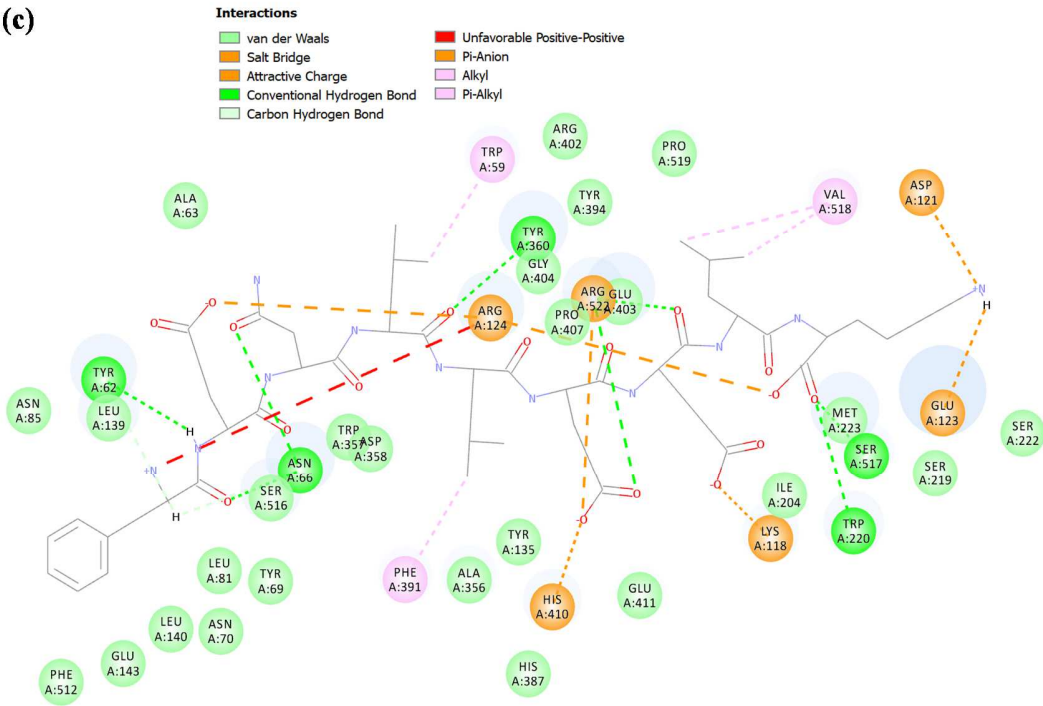


Fig. 3

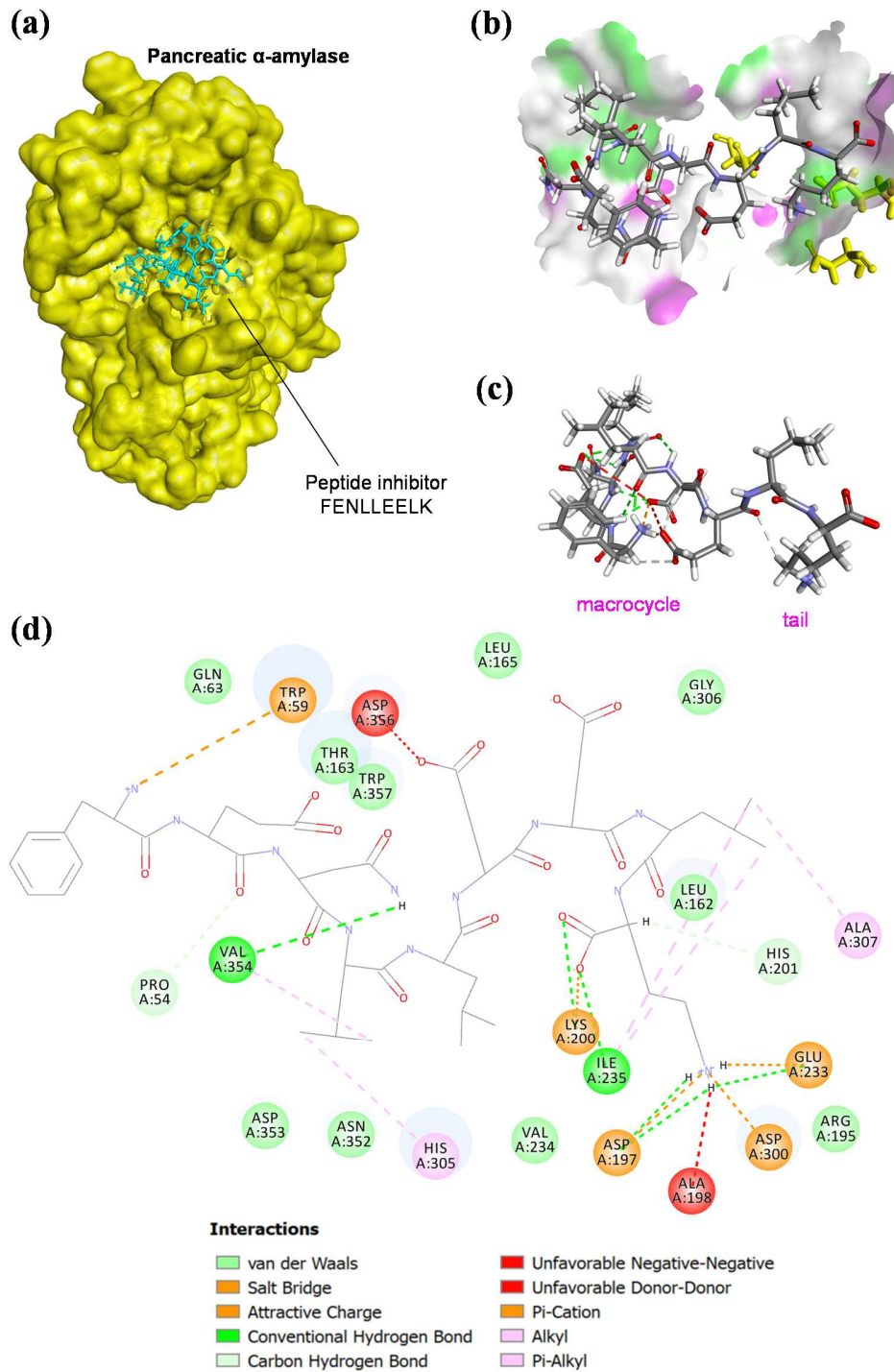


Fig. 4

