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Abstract

Recently, interest in food-derived bioactive peptides as promising ingredients for the prevention and improvement of hypertension is increasing. The purpose of this study was to determine the structure and antihypertensive effect of an antioxidant peptide purified from velvet antler in a previous study and evaluate its potential as a various bioactive peptide. Molecular weight (MW) and amino acid sequences of the purified peptide were determined by Q-TOF ESI mass spectroscopy. The angiotensin I-converting enzyme (ACE) inhibition activity of the purified peptide was assessed by enzyme reaction methods and in silico molecular docking analysis to determine the interaction between the purified peptide and ACE. Also, antihypertensive effect of the purified peptide in spontaneously hypertensive rats (SHRs) was investigated. The purified antioxidant peptide was identified to be a pentapeptide Asp-Asn-Arg-Tyr-Tyr (DNRYY) with a MW of 730.31 Da. This pentapeptide showed potent inhibition activity against ACE (IC_{50} value, 3.72 μM). Molecular docking studies revealed a good and stable binding affinity between purified peptide and ACE and indicated that the purified peptide could interact with HOH2570, ARG522, ARG124, GLU143, HIS387, TRP357, and GLU403 residues of ACE. Furthermore, oral administration of the pentapeptide significantly reduced blood pressure in SHRs. The pentapeptide derived from enzymatic hydrolysate of velvet antler is an excellent ACE inhibitor. It might be effectively applied as an animal-based functional food ingredient.

Keywords: velvet antler, angiotensin I-converting enzyme, purified peptide, antihypertensive effect, animal-based functional food ingredients

Introduction

Hypertension is a severe chronic disease. It is the biggest contributor to stroke and coronary heart disease (Mills et al., 2020). Thus, hypertension is a major cause of premature death worldwide. Hypertension is regulated by several mechanisms including modulating of baroreceptor reflex, antidiuretic hormone, and renin-angiotensin-aldosterone system (RAAS) in human body (Brooks, 1997; Takahashi et al., 2011). Among these mechanisms, the RAAS is an essential factor of blood pressure regulation. It can increase blood volume and systemic vascular resistance (Pugliese et al., 2020). RAAS is mainly regulated through mediating bradykinin and angiotensin converting enzyme (ACE) (Pugliese et al., 2020). ACE plays a critical physiological role in regulating blood pressure by converting angiotensin-I to angiotensin-II in the RAAS (Bernstein et al., 2014; Qian et al., 2019). Angiotensin II converted by ACE has many functions, including potent vasoconstriction action, release of antidiuretic hormone and aldosterone, and increased sodium reabsorption, thus increasing blood pressure (Bernstein et al., 2014; Chen et al., 2009; Qian et al., 2019). Thus, ACE inhibition activity is considered to be a major strategy in the prevention and treatment of hypertension (Lee and Hur, 2017; Miralles et al., 2018; Zhao et al., 2019).

Synthetic ACE inhibitor drugs such as enalapril, captopril, and benazepril have certain side effects such as headaches, insomnia, fatigue, and increased blood potassium levels (Lee and Hur, 2017). However, ACE inhibitors derived from natural products are stable and have minimal side effects compared with abovementioned synthetic ACE inhibitor. Therefore, over the last few decades, the discovery of natural ACE inhibitors has become a major research field (Chen et al., 2009; Iwaniak et al., 2014; Lee and Hur, 2017). Especially, ACE inhibitory peptides derived from various foods have gained increased research interest worldwide.

Recently, many bioactive peptides with ACE inhibition activity have been successfully isolated from protein-rich foods (animal and non-animal protein sources) (Lee and Hur, 2017; Miralles et al., 2018; Toldrá et al., 2020). It has proven that they can be used as potential functional food ingredients to prevent and improve hypertension (Gallego et al., 2018; Liao et al., 2021).

Velvet antler is called Nokyong in Korea. It has been used as a traditional medicinal ingredient of animal origin for over 2000 years in Asia. It is still recognized as one of the powerful animal-based traditional medicine in pharmacopeias of Korea and China (Ding et al., 2019; Sui et al., 2014). Nowadays, in places such as East Asia, USA, Canada, and New Zealand, it is also used as a functional food ingredient and a health food supplement to prevent various diseases (Wu et al., 2013). Recently, numerous studies have reported that bioactive peptides and enzymatic hydrolysate derived from velvet antler show a various biological activities (Ding et al., 2019; Ding et al., 2017; Lee et al., 2015; Zhao et al., 2011; Zhao et al., 2016). However, as far as we know, there have been rarely reports about antihypertensive peptide derived from velvet antler by enzymatic hydrolysis used in this study. In our previous study, the peptide purified from alcalase hydrolysate of velvet antler was found to possess potent antioxidant activity through a peroxyl radical scavenging activity (Ding et al., 2019). However, the structure of the purified antioxidant peptide and its antihypertensive effects remain to be elucidated.

Therefore, the objective of the current study was to present the evidence for the antihypertensive effect of an antioxidant peptide purified from velvet antler alcalase hydrolysate, and verify its potential as a multi-functional bioactive peptide for use as a health food ingredient. Herein the present research investigated the structure characterization, ACE inhibitory activity, and potential interactions on ACE of the purified peptide. Furthermore, we have also investigated the antihypertensive action by oral administration in SHRs.

Materials and Methods

Preparation of velvet antler sample

Velvet antler collected from a farmed elk at 75 days after casting was obtained from Daesungsan Deer Farm (Daegwallyeong, Korea). Sliced fresh velvet antler was freeze-dried and ground into a fine powder.

Structure characterization of purified antioxidant peptide

To verify the antihypertensive effect of antioxidant peptide derived from velvet antler, separation and purification of antioxidant peptide were performed according to a previously reported method (Ding et al., 2019). Firstly, 10 g of the ground dried velvet antler powder was added into 1 L of distilled water. Alcalase enzyme (food-grade commercial *proteases,* Novo Nordisk, Bagsvaerd, Denmark) was then added with a substrate to enzyme ratio of 100:1.Enzymatic hydrolysis was conducted under optimal conditions (pH 8.0, 50°C) for 24 h, after which the hydrolysate was boiled at 100°C for 10 min to inactivate the enzyme. The hydrolysate was clarified by centrifugation at 3000 × *g* for 20 min to remove any unhydrolyzed residue. The supernatant of alcalase hydrolysate was filtered and adjusted to pH 7.0. The enzymatic hydrolysate was fractionated using ultrafiltration membranes (MWCO: 5 kDa) with a Millipore's Lab scale TFF system (Millipore Corporation, Bedford, MA, USA) at 4 ℃. Then, < 5 kDa fraction was obtained and lyophilized. The fraction (500 mg) was separated with ultrafiltration membranes and dissolved with 5 ml of filtered distilled water. This fraction was loaded onto a Sephadex G-25 column (2.5 × 100 cm) pre-equilibrated with filtered distilled water. Elution was then carried out with filtered distilled water at a flow rate of 1.5 ml/min.

Absorbance of each fraction was measured at 220 nm and sub-fractions were collected. Lastly, the target fraction was then subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on an Atlantis T3 column (3 μm, 3.0 × 150 mm, Waters, NY, USA) with a linear gradient of acetonitrile (0-100% v/v, 30 min) at a flow rate of 1.0 ml/min. Elution peaks were detected at 220 nm. Finally, the antioxidant peptide was collected and lyophilized followed by identification of its amino acid sequences.

Identification of purified antioxidant peptide

Molecular weight and amino acid sequences of the antioxidant peptide purified from velvet antler were determined using a MicroQ-TOFIII mass spectrometer (Bruker Daltonics, 255748 Germany) coupled with an electrospray ionization (ESI) source. The purified peptide was dissolved in distilled water and infused into the ESI source. Its molecular weight was determined by singly charged (M+H) state analysis in mass spectrum.

Assay of ACE inhibition activity

ACE inhibition activity of antioxidant peptide was measured using an ACE kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in accordance with the manufacturer's protocols. This ACE kit-WST assay is safe and simple. It provides highly reproducible data.

In silico molecular docking analysis of ACE

In order to assess the interaction between DNRYY and ACE, molecular docking analysis were

carried out using Accelrys Discovery Studio 3.5 (Accelrys, Inc., San diego, CA, USA), according to a previously reported procedure (Kang et al., 2020). The crystal structure of ACE (PDB code: 1O86) was obtained from the Protein Data Bank (PDB, http://www.rcsb.org) and then molecular docking of DNRYY into the active site of the ACE was performed. The molecular docking runs were performed with the CDOCKER and Calculate Binding Energies tool. Docking of DNRYY to ACE was performed as follows: (1) a 2D structure was converted into a 3D structure; (2) proteins were prepared, and the binding site was defined; and (3) docking of compounds was performed using CDOCKER tool. The binding energy of the produced candidate compound-protein complexes were calculated using Calculate Binding Energies tool.

Anti-hypertensive effect in animal model

Anti-hypertensive effect in animal model was carried out in accordance with a previously reported method (Ko et al., 2012). Male spontaneously hypertensive rats (SHRs, 10 weeks of age) were purchased from SLC, Inc., (Shizuoka, Japan). SHRs with tail systolic blood pressure (SBP) over 180 mmHg were randomly divided into three groups (n = 6): control group (saline); sardine peptide treated group (positive control); and the purified peptide treated group. Saline, sardine peptide, and the purified peptide dissolved in 1 mL saline were orally administered to SHRs at a dose of 50 mg/kg. SBP was measured with a tail-cuff method at 0, 3, 6, and 9 h post-administration using a CODA^{TM} blood pressure monitor (Kent Scientific Corp., Torrington, CT, USA). The experimental protocol was approved by the Laboratory Animal Administration Committee of Jeju National University (Approval NO. 2017-0017). The experiment was conducted according to Animal Experimentation Guidelines of the University.

Statistical Analysis

The results are represented as mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance (ANOVA) complemented by Duncan's multiple range test using SPSS software version 28 (IBM Corp., Armonk, NY, USA). The level of statistical significance was set at $P < 0.05$.

Results and Discussion

Structure characterization of the purified antioxidant peptide

In our previous study, the IC_{50} value of the purified peptide from Alcalase hydrolysate of velvet antler against peroxyl radical was 0.343 mg/mL, suggesting that the purified peptide could possess potent antioxidant activity (Ding et al., 2019). As previous reported, molecular weight (MW) and structure of the bioactive peptides is closely related to ACE inhibitory activity (Lee and Hur, 2017). However, the MW and amino acid sequence of the purified peptide have not been determined yet. Thus, in this study, amino acid sequence and MW of the purified peptide were determined using a Q-TOF ESI mass spectrometer. As shown in Figure 1, the purified antioxidant peptide was identified as a pentapeptide Asp-Asn-Arg-Tyr-Tyr (named DNRYY). The MW of this pentapeptide was 730.31 Da.

ACE inhibition activity of the antioxidant peptide

Antihypertensive effect is the most extensively studied bioactivity in relation to food-derived peptides. They main mechanism of action is based on the inhibition of the ACE that converts the inactive angiotensin I into the potent vasoconstricting angiotensin II (Miralles et al., 2018).

Angiotensin II produced by the action of ACE also amplifies oxidative stress by inducing the formation of intracellular reactive oxygen species (Del Pino-García et al., 2017; Kang et al., 2020). Thus, ACE inhibition intensifies the antioxidant defense system in humans. Several food-derived bioactive peptides have been reported to possess both antioxidant and antihypertensive effects (Kang et al., 2020; Toldrá et al., 2020). Therefore, we investigated to provide evidence for the antihypertensive effect of an antioxidant peptide purified from velvet antler alcalase hydrolysate in the present study. In order to validate the antihypertensive effect of the purified antioxidant peptide (DNRYY), we evaluated the inhibitory activity of DNRYY against ACE. As shown in Figure. 2, DNRYY inhibited ACE activity in a dose dependent manner. At concentrations of 1.75, 3.5, 7, and 14 μM, it inhibited ACE activity by 23.56%, 48.11%, 69.83%, and 89.53%, respectively. IC_{50} values of DNRYY against ACE was 3.72 μM. Findings of this study exhibited that DNRYY might be useful as an effective velvet antler-derived antihypertensive peptide. The structure of a peptide is an important factor for its ACE inhibitory activity (Lee et al., 2014; Lee et al., 2009). As reported previously, aromatic amino acids at the C-terminus, hydrophobic amino acid residues at the N-terminus, and positively charged amino acids at the middle in peptide structure play an important role in the ACE inhibition activity of peptides (Cushman and Cheung, 1971, Welderufael et al., 2012; Wu et al., 2016). Especially, it has been proven that peptides with C-terminal Tyr residues such as Val-Tyr, Met-Tyr, Ile-Tyr, and Arg-Val-Tyr show potent ACE inhibitory activities (Chen et al., 2009; Erdmann et al., 2006; Ko et al., 2012; Liu et al., 2021; Yu et al., 2019). In this study, the identified pentapeptide contained Tyr at the C-terminal, which might have contributed to its ACE inhibition activity. This study provides strong evidence for ACE inhibitory activity of antioxidant peptide purified from velvet antler alcalase hydrolysate. Results of preceding studies and the present study demonstrated that the purified peptide had both antioxidant and

191 antihypertensive effects.

192

193 **In silico molecular docking of ACE**

194 Molecular docking is an application wherein molecular modeling techniques are used to
195 predict how a protein (enzyme) interacts with small molecules (ligands) (Ko and Lee, 2021).
196 The application of in silico molecular docking tools can simplify process and cut down costs
197 for discovery of bioactive peptides, and illuminate the action mechanism (Zhao et al., 2019;
198 Fan et al., 2020). In the present study, in order to understand potential molecular interactions
199 between the velvet antler-derived pentapeptide (DNRYY) structure and specific amino acids at
200 the binding site of ACE, computational molecular docking of the DNRYY into the binding site
201 of ACE (PDB code: 1O86) was conducted using Discovery Studio 3.5 (DS). As shown in
202 Figure. 3A, the complex of ACE-DNRYY binding sites was predicted by the 2D diagram.
203 DNRYY formed five hydrogen bonds with amino acid residues in ACE: one with HOH2570,
204 one with ARG522, one with ARG124, and two with GLU143. DNRYY also formed two pi
205 interactions with HIS387 and TRP357 residues in ACE. Additionally, DNRYY interacted with
206 the GLU403 (attractive charge) and ARG522 (salt bridge) amino acid residues through
207 electrostatic interactions. These results suggest that DNRYY can interact with amino acids
208 residues of ACE and inhibit ACE activity. Pi interactions and Hydrogen bonds in ACE-ligand
209 interactions are known to contribute to ACE inhibition activity (Fu et al., 2017; Ling et al.,
210 2018; Tu et al., 2018). It has been reported that food-derived bioactive peptides have strong
211 interactions with ACE by forming high numbers of pi interactions and hydrogen bonds with
212 ACE amino acid residues, thus effectively inhibiting ACE (Chamata et al., 2020; Fan et al.,
213 2020; Ko et al., 2017). The present study also showed that DNRYY could form high numbers

of pi interactions and hydrogen bonds with ACE amino acid residues. Accordingly, our results suggest that the potent ACE inhibitory activity of DNRYY can be attributed to high numbers of hydrogen bonds and pi interactions at the ACE binding site. The docking of stabilized poses of ACE-DNRYY complex was obtained from DS 3.5 binding energy program. Binding energy value was -86.200 kcal/mol for CDOCKER interaction energy and -456.853 kcal/mol for binding energy. These results indicate that DNRYY has a good and stable binding affinity with ACE. Overall, the docking results provide strong evidence for the ACE inhibition activity of DNRYY.

Antihypertensive effect of ACE inhibitory peptide (DNRYY) in SHRs

ACE-inhibitory peptides derived from foods have gained interest as potential hypertension treatment agents. Several studies have reported that bioactive peptides exhibited significant blood pressure lowering effect by inhibiting ACE activity *in vivo* studies using SHRs (Lee and Hur, 2017; Miralles et al., 2018; Toldrá et al., 2020). Thus, to verify the *in vivo* antihypertensive effect of ACE inhibitory peptide (DNRYY) derived from velvet antler alcalase hydrolysate, the ACE inhibitory peptide was evaluated by measuring changes of systolic blood pressure (SBP) at 3, 6, and 9 h after oral administration of 50 mg/kg of body weight to SHRs. The SBP reduction effect of DNRYY was compared with effects of a sardine peptide, a commercially available antihypertensive peptide (derived from *Sardinops melanostictus*) used in blood pressure lowering functional food ingredient in Korea and Japan. As shown in Figure 4, the saline administrated group exhibited slight changes in SBP in the range of 185–197 mmHg. However, at 3 h after oral administration of DNRYY, SBP was significantly decreased compared to that in the saline group. The DNRYY administrated group showed a similar SBP

reduction to the sardine peptide administrated group with activities maintained until 6 hours. These results clearly show that oral administration of purified peptide (DNRYY) can decrease SBP significantly in SHRs. Previous many studies have revealed that food-derived peptides consisting of Tyr at the C-terminus, such as Val-Tyr, Met-Tyr, Arg-Val-Tyr, Ile-Tyr, and Val-Glu-Gly-Tyr show antihypertensive effects in SHRs through ACE inhibition activity (Ko et al., 2012; Matsui et al., 2004; Tanaka et al., 2006). Accordingly, the antihypertensive effect of DNRYY in SHRs can be attributed to its ACE inhibition activity. These results of this study confirmed that this ACE inhibitory peptide from velvet antler alcalase hydrolysate could effectively reduction of blood pressure.

Conclusion

In this study, the structure and antihypertensive effect of an antioxidant peptide derived from Alcalase hydrolysate of velvet antler were investigated. The identified pentapeptide (Asp-Asn-Arg-Tyr-Tyr, 730.31 Da) was demonstrated to be a great ACE inhibitory peptide based on ACE inhibition activity assay and in silico molecular docking analysis. In addition, oral administration of this pentapeptide significantly reduced SBP in SHRs. These results suggest that the antihypertensive effect of this pentapeptide might contribute to inhibition of ACE. Therefore, this ACE inhibitory peptide derived from velvet antler is a multiple bioactive peptide with potential applications as animal-based functional food ingredients and health food supplement. Nevertheless, gastrointestinal digestion by oral administration of bioactive peptides have demonstrated to affect the physiological action and, therefore, should also be considered. Therefore, further investigations are needed on the resistant to gastrointestinal digestion of ACE inhibitory peptide derived from velvet antler.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References

Bernstein KE, Giani JF, Shen XZ, Gonzalez-Villalobos RA. 2014. Renal angiotensin-converting enzyme and blood pressure control. Curr Opin Nephrol Hypertens 23:106-112.

Brooks VL. 1997. Interactions between angiotensin II and the sympathetic nervous system in the the long-term control of arterial pressure. Clin Exp Pharmacol Physiol 24:83-90.

Chamata Y, Watson KA, Jauregi P. 2020. Whey-derived peptides interactions with ACE by molecular docking as a potential predictive tool of natural ACE Inhibitors. Int J Mol Sci 21:864.

Chen ZY, Peng C, Jiao R, Wong YM, Yang N, Huang Y. 2009. Anti-hypertensive nutraceuticals and functional Foods. J Agric Food Chem 57:4485–4499.

Cushman DW, Cheung HS. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20:1637-1648.

Del Pino-García R, Rivero-Pérez MD, González-SanJosé ML, Croft KD, Muñiz P. 2017. Antihypertensive and antioxidant effects of supplementation with red wine pomace in spontaneously hypertensive rats. Food Funct 8:2444–2454.

299 Ding Y, Ko SC, Moon SH, Lee SH. 2019. Protective effects of novel antioxidant peptide
300 purified from Alcalase hydrolysate of velvet antler against oxidative stress in Chang liver cells
301 in vitro and in a zebrafish model in vivo. Int J Mol Sci 20:5187.

302

303 Ding Y, Wang Y, Jeon BT, Moon SH, Lee SH. 2017. Enzymatic hydrolysate from velvet antler
304 suppresses adipogenesis in 3T3-L1 cells and attenuates obesity in high-fat diet-fed mice.
305 EXCLI J 22:328-339

306

307 Erdmann K, Grosser N, Schipporeit K, Schröder H. 2006. The ACE inhibitory dipeptide Met-
308 Tyr diminishes free radical formation in human endothelial cells via induction of heme
309 oxygenase-1 and ferritin. J Nutr 136:2148-2152.

310

311 Fan Y, Yu Z, Zhao W, Ding L, Zheng F, Li J, Liu J. 2020. Identification and molecular
312 mechanism of angiotensin-converting enzyme inhibitory peptides from *Larimichthys crocea*
313 titin. Food Sci Hum Wellness 9:257-263.

314

315 Fu Y, Alashi AM, Young JF, Therkildsen M, Aluko RE. 2017. Enzyme inhibition kinetics and
316 molecular interactions of patatin peptides with angiotensin I-converting enzyme and renin. Int
317 J Biol Macromol 101:207-213.

318

Gallego M, Mora Leticia, Toldrá F. 2018. Health relevance of antihypertensive peptides in foods. Curr Opin Food Sci 19:8-14.

Iwaniak, A., Minkiewicz, P. and Darewicz, M. 2014. Food-originating ACE Inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. Compr Rev Food Sci Food Saf 13:114-34.

Kang N, Ko SC, Kim HS, Yang HW, Ahn G, Lee SH, Lee TG, Lee JS, Jeon YJ. 2020. Structural evidence for antihypertensive effect of an antioxidant peptide purified from the edible marine animal *Styela clava*. J Med Food 23:132-138.

Ko SC, Kang N, Kim EA, Kang MC, Lee SH, Kang SM, Lee JB, Jeon BT, Kim SK, Park SJ, Park PJ, Jung WK, Kim D, Jeon YJ. 2012. A novel angiotensin I-converting enzyme (ACE) inhibitory peptide from a marine *Chlorella ellipsoidea* and its antihypertensive effect in spontaneously hypertensive rats. Process Biochem 47:2005-2011.

Ko SC, Jang J, Ye BR, Kim MS, Choi IW, Park WS, Heo SJ, Jung WK . 2017. Purification and molecular docking study of angiotensin I-converting enzyme (ACE) inhibitory peptides from hydrolysates of marine sponge *Stylotella aurantium*. Process Biochem 54:180-187.

Ko SC, Lee SH. 2021. Protocatechuic aldehyde inhibits α-MSH-induced melanogenesis in

340 B16F10 melanoma cells via PKA/CREB-associated MITF downregulation. Int J Mol Sci 22:
341 3861.

342

343 Lee JK, Jeon JK, Byun HG. 2014. Antihypertensive effect of novel angiotensin I converting
344 enzyme inhibitory peptide from chum salmon (*Oncorhynchus keta*) skin in spontaneously
345 hypertensive rats. J Funct Foods 7:381-389.

346

347 Lee JK, Hong S, Jeon JK, Kim SK, Byun HG. 2009. Purification and characterization of
348 angiotensin I converting enzyme inhibitory peptides from the rotifer, *Brachionus rotundiformis*.
349 Bioresour Technol 100:5255-5259.

350

351 Lee SH, Yang HW, Ding Y, Wang Y, Jeon YJ, Moon SH, Jeon BT, Sung SH. 2015. Anti-
352 inflammatory effects of enzymatic hydrolysates of velvet antler in RAW 264.7 cells in vitro
353 and zebrafish model. EXCLI J 20:1122-1132.

354

355 Lee SY, Hur SJ. 2017. Antihypertensive peptides from animal products, marine organisms, and
356 plants. Food Chem 228:506-517.

357

358 Liao W, Sun G, Xu D, Wang Y, Lu Y, Sun J, Xia H, Wang S. 2021. The blood-pressure-lowering
359 effect of food-protein-derived peptides: A meta-analysis of recent clinical trials. Foods 10:2316.

Ling Y, Liping S, Yongliang Z. 2018. Preparation and identification of novel inhibitory angiotensin-I-converting enzyme peptides from tilapia skin gelatin hydrolysates: inhibition kinetics and molecular docking. Food Funct 9:5251-5259.

Liu WY, Zhang JT, Miyakawa T, Li GM, Gu RZ, Tanokura M. 2021. Antioxidant properties and inhibition of angiotensin-converting enzyme by highly active peptides from wheat gluten. Sci Rep 11:5206.

Matsui T, Imamura M, Oka H, Osajima K, Kimoto KI, Kawasaki T, Matsumoto K. 2004. Tissue distribution of antihypertensive dipeptide, Val-Tyr, after its single oral administration to spontaneously hypertensive rats. J Pept Sci 10:535-545.

Mills KT, Stefanescu A, He J. 2020. The global epidemiology of hypertension. Nat Rev Nephrol 16:223-237.

Miralles B, Amigo L, Recio I. 2018. Critical review and perspectives on food-derived antihypertensive peptides. J Agric Food Chem 66: 9384-9390.

Pugliese NR, Masi S, Taddei S. 2020. The renin-angiotensin-aldosterone system: a crossroad from arterial hypertension to heart failure. Heart Fail Rev 25:31-42.

Qian B, Tian C, Huo J, Ding Z, Xu R, Zhu J, Yu L, Villarreal OD. 2019. Design and evaluation of four novel tripeptides as potent angiotensin converting enzyme (ACE) inhibitors with anti-hypertension activity. Peptides 122:170171.

Sui Z, Zhang L, Huo Y, Zhang Y. 2014. Bioactive components of velvet antlers and their pharmacological properties. J Pharm Biomed Anal 87:229-240.

Takahashi H, Yoshika M, KomiyamaY, Nishimura M. 2011. The central mechanism underlying hypertension: a review of the roles of sodium ions, epithelial sodium channels, the renin–angiotensin–aldosterone system, oxidative stress and endogenous digitalis in the brain. Hypertens Res 34:1147–60.

Tanaka M, Matsui T, Ushida Y, Matsumoto K. 2006. Vasodilating effect of di-peptides in thoracic aortas from spontaneously hypertensive rats. Biosci Biotechnol Biochem 70:2292-2295.

Toldrá F, Gallego M, Reig M, Aristoy MC, Mora L. 2020. Recent progress in enzymatic release of peptides in foods of animal origin and assessment of bioactivity. J Agric Food Chem 68:12842-12855.

Tu M, Wang C, Chen C, Zhang R, Liu H, Lu W, Jiang L, Du M. 2018. Identification of a novel

401 ACE-inhibitory peptide from casein and evaluation of the inhibitory mechanisms. Food Chem
402 256:98-104.

403

404 Welderufael FT, Gibson T, Methven L, Jauregi P. 2012. Chemical characterisation and
405 determination of sensory attributes of hydrolysates produced by enzymatic hydrolysis of whey
406 proteins following a novel integrative process. Food Chem 134:1947-1958.

407

408 Wu F, Li H, Jin L, Li X, Ma Y, You J, Li S, Xu Y. 2013. Deer antler base as a traditional Chinese
409 medicine: a review of its traditional uses, chemistry and pharmacology. J Ethnopharmacol
410 145:403-415.

411

412 Wu Q, Du J, Jia J, Kuang C. 2016. Production of ACE inhibitory peptides from sweet sorghum
413 grain protein using alcalase: Hydrolysis kinetic, purification and molecular docking study.
414 Food Chem 199:140-149.

415

416 Yu D, Wang C, Song Y, Zhu J, Zhang X. 2019. Discovery of novel angiotensin-converting
417 enzyme inhibitory peptides from *Todarodes pacificus* and their inhibitory mechanism: In silico
418 and in vitro studies. Int J Mol Sci 20:4159.

419

Zhao L, Luo YC, Wang CT, Ji BP. 2011. Antioxidant activity of protein hydrolysates from aqueous extract of velvet antler (*Cervus elaphus*) as influenced by molecular weight and enzymes. Nat Prod Commun 6:1683-1688.

Zhao L, Wang X, Zhang XL, Xie QF. 2016. Purification and identification of anti-inflammatory peptides derived from simulated gastrointestinal digests of velvet antler protein (*Cervus elaphus* Linnaeus). J Food Drug Anal 24:376-384.

Zhao W, Xue S, Yu Z, Ding L, Li J, Liu J. 2019. Novel ACE inhibitors derived from soybean proteins using in silico and in vitro studies. J Food Biochem 43:e129

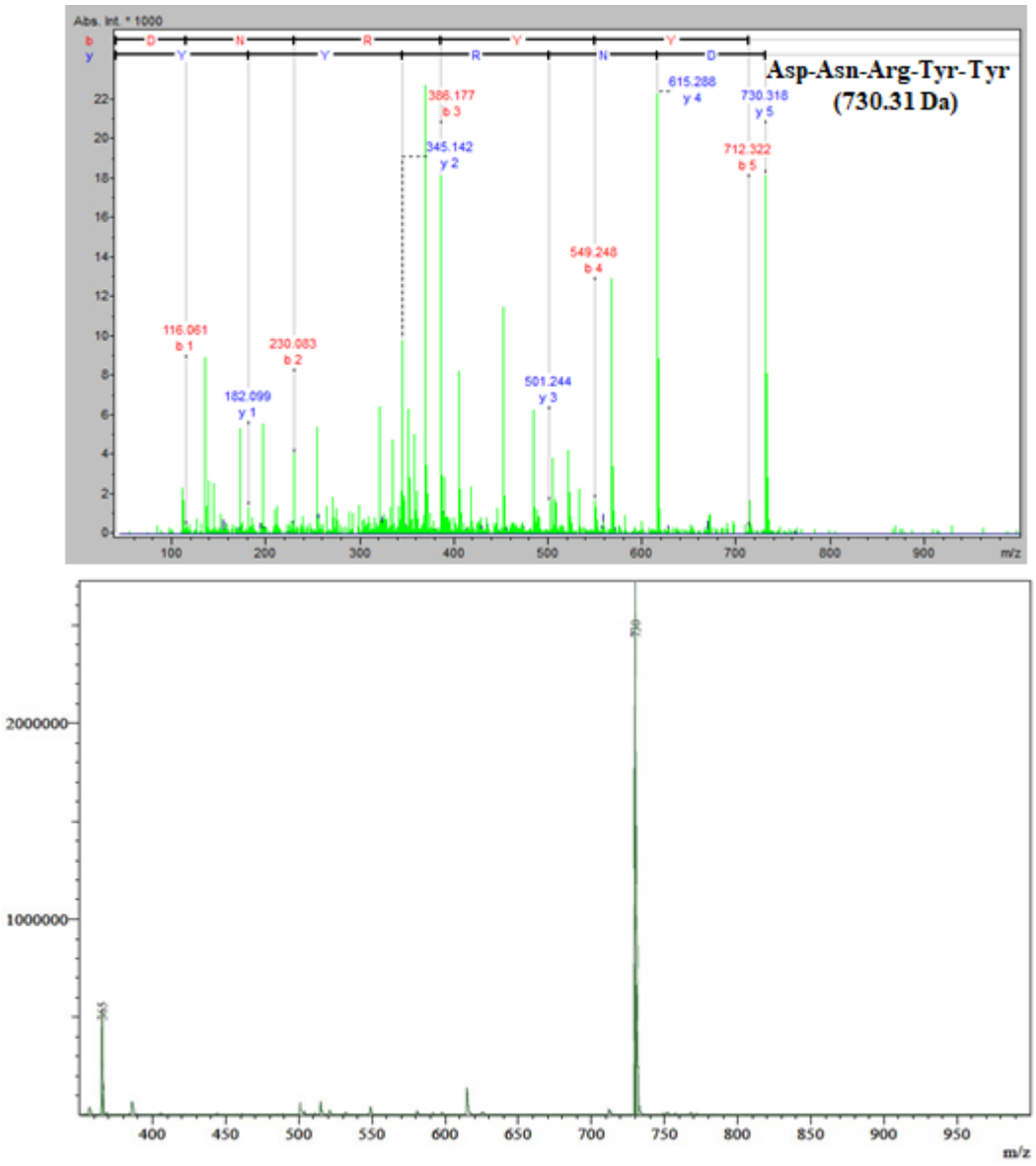
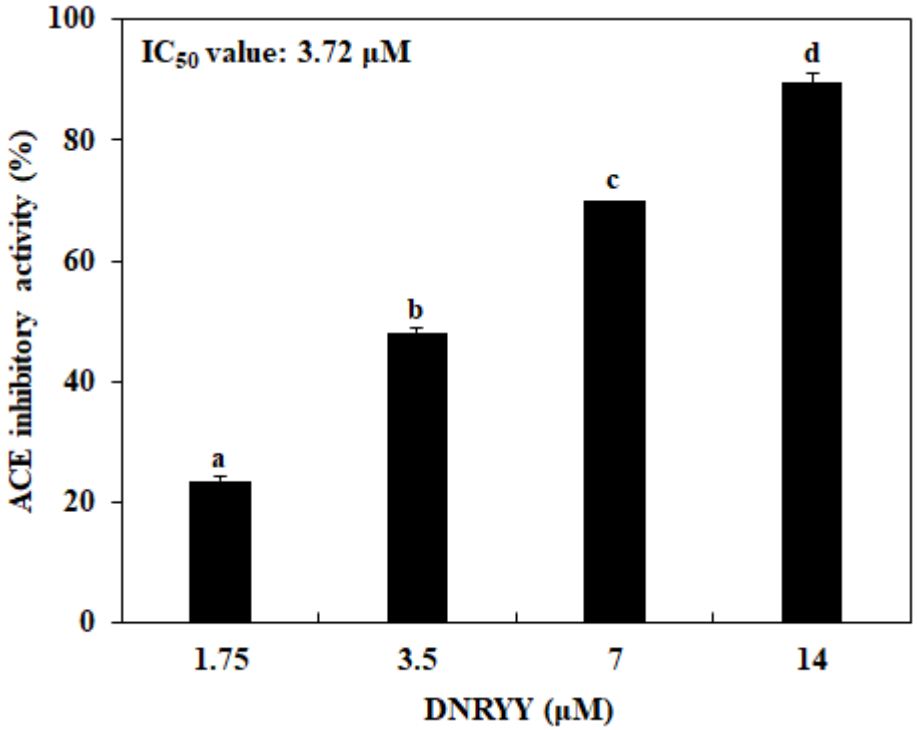


Fig. 1. Identification of amino acid sequence (upper panel) and molecular weight (lower panel) of the antioxidant purified peptide from alcalase hydrolysate of velvet antler by Q-TOF ESI mass spectrometer.



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448 **Fig. 2.** ACE inhibitory activity of DNRYY. Inhibitory activity was measured using an ACE kit.

449 Values are expressed as mean ± SD of triplicate experiments. [a-d]Values with different letters are

450 significantly different at $P < 0.05$, as determined by Duncan's multiple range test.

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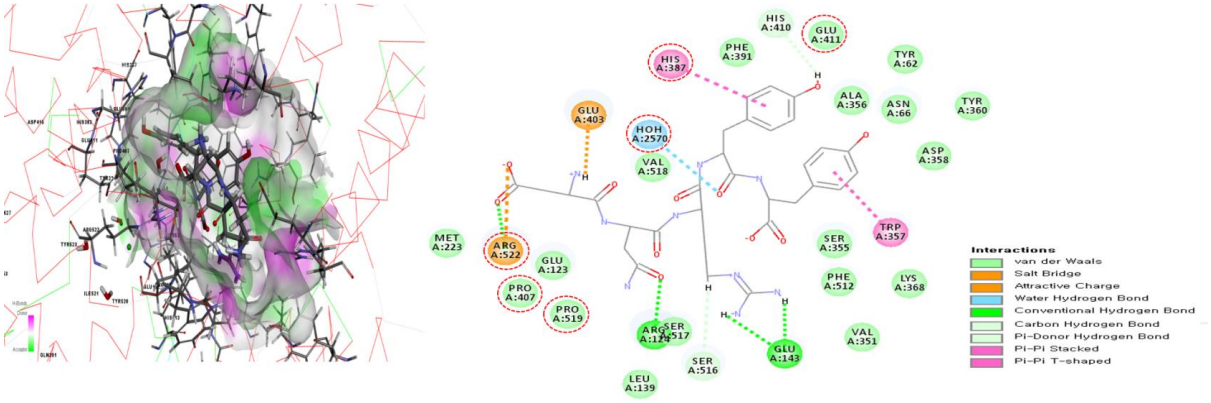
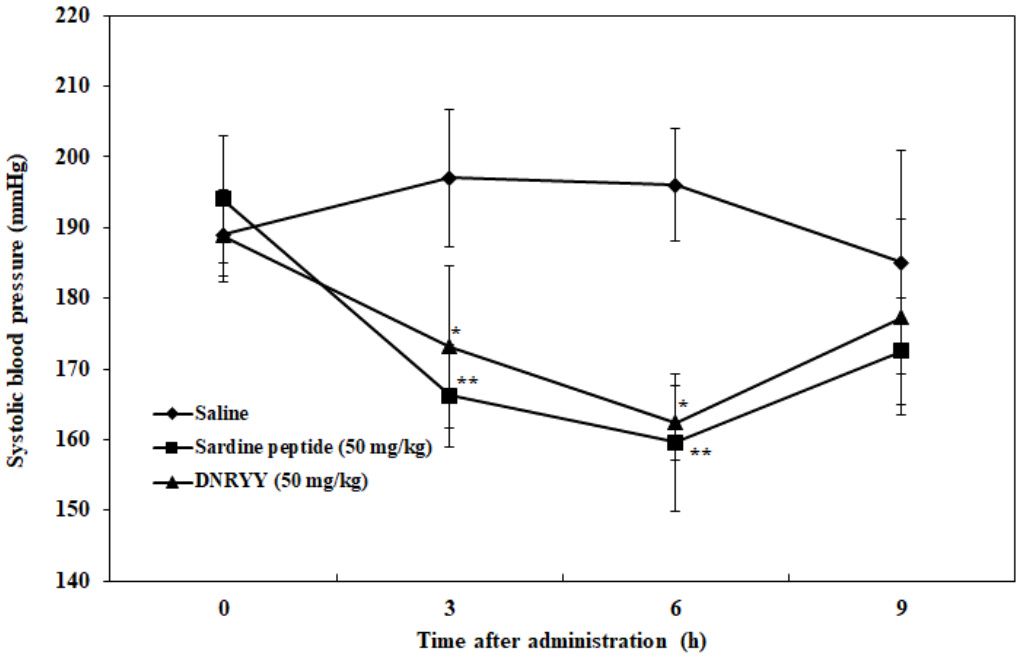
(A)



(B)

ACE-DNRYY complex	
CDOCKER interaction energy (kcal/mol)	-86.2008
Binding energy (kcal/mol)	-456.853

Fig. 3. Specific interactions between DNRYY and ACE after automated docking of DNRYY to the ACE enzyme binding site. Predicted 3D structure of the ACE (Protein Data Bank; PDB code: 1O86)–DNRYY complex and 2D diagram (A) are shown. Binding energy values were obtained from Discovery Studio (DS) 3.0 binding energy calculation program (B).



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468 **Fig. 4.** Change of SBP after oral administration of ACE inhibitory peptide (DNRYY) in SHR.
469 Sardine peptide was used as a positive control. Single oral administration was performed at a
470 dose of 50 mg/kg body weight. SBP was measured at 0, 3, 6, and 9 h after oral administration
471 of DNRYY. Values are expressed as mean ± SD of triplicate experiments. *, $P < 0.05$; **$P <$
472 0.01, indicate significant differences compared with the only saline-treated control group.

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