Journal of Proteomics 128 (2015) 424–435

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Screening, discovery, and characterization of angiotensin-I converting enzyme inhibitory peptides derived from proteolytic hydrolysate of bitter melon seed proteins

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ARTICLE INFO

Article history:
Received 3 August 2015
Received in revised form 24 August 2015
Accepted 27 August 2015
Available online 3 September 2015

Keywords:
ACE inhibitory peptide
Bitter melon seed protein
Momordin a
Molecular docking
LC-MS/MS
Momordica charantia

ABSTRACT

In this study, new angiotensin-I converting enzyme (ACE) inhibitory peptides were comprehensively identified from a thermolysin digest of bitter melon (*Momordica charantia*) seed proteins. The hydrolysate was fractionated by reversed-phase high performance liquid chromatography (RP-HPLC), and the inhibitory activities of the resulting fractions were evaluated using ACE inhibitory assay. Two novel ACE inhibitory peptides (VY-7 and VG-8) were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database-assisted peptide sequencing. VY-7 and VG-8 were derived from momordin A and MAP30, respectively, and their IC_{50} values were as low as 8.64 ± 0.60 and 13.30 ± 0.62 μM, respectively. Lineweaver–Burk plots further indicated that VY-7, which showed the best IC_{50} value, acts as a competitive inhibitor. Notably, the content of VY-7 in crude thermolysin digest was determined to be as high as 14.89 ± 0.88 μg/mg using LC-MS/MS quantification. In the spontaneously hypertensive rat (SHR) model, oral administration of VY-7 at 2 mg/kg body weight significantly decreased the systolic blood pressure. The interaction between VY-7 and ACE was examined using molecular docking calculations and the results suggested that certain residues of VY-7 can fit perfectly into the S1, S1' and S2' regions of the binding pocket of ACE.

Biological significance: One of the most common supportive therapies for treating hypertension is the use of synthetic drugs to inhibit ACE activity. Synthetic ACE inhibitors possess good antihypertensive effects, but come with accompanying side effects. Therefore, food-derived ACE inhibitory peptides are regarded as safer alternatives and are attracting much attention for hypertension treatment. In this study, we comprehensively identified peptides derived from bitter melon (*Momordica charantia*) seed proteins (BMSPs) using a shotgun proteomics approach. Based on results from an *in vitro* ACE inhibitory assay, two peptides (VY-7 and VG-8) derived from momordin A and MAP30 proteins, respectively, showed good ACE inhibitory activities. For VY-7, which showed the best IC_{50} value (8.64 ± 0.60 μM), the inhibition type was determined to be competitive inhibition, as found using a Lineweaver–Burk plot. The novel ACE inhibitory peptide VY-7 (at 2 mg/kg body weight) as well as the crude hydrolysate of BMSPs (at 10 mg/kg body weight) showed significant and moderate antihypertensive effects, respectively, in an animal model of hypertension, spontaneously hypertensive rats (SHRs). The present work demonstrated the screening of ACE inhibitory peptides from BMSPs and, as far as we know, VY-7 is the first well-characterized antihypertensive peptide derived from bitter melon seeds.

1. Introduction

Cardiovascular disease (CVD) has become a prominent contributor to mortality in many countries, especially in developing ones [1]. CVD is caused by physiological and morphological changes of the heart and blood vessels, which alter the cardiovascular function and lead to several heart and circulatory diseases, such as coronary heart disease (angina

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http://dx.doi.org/10.1016/j.jprot.2015.08.018
1874-3919/

and heart attack), heart failure, congenital heart disease and stroke [2]. High blood pressure (HBP) is a primary risk factor of CVD. It is estimated that more than half of CVDs are associated with HBP [3]. Pharmacological studies classify antihypertensive drugs based on their mechanisms of action, such as angiotensin-I converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, calcium channel blockers, beta blockers, and diuretics [4]. Among them, ACE inhibitors are commonly used and recommended to treat HBP especially in high risk CVD patients [5–7]. The renin-angiotensin-aldosterone system (RAAS) plays a vital role in blood pressure regulation because ACE catalyzes the conversion of the decapeptide angiotensin I to the strong vasoconstrictor octapeptide angiotensin II [8]. Meanwhile, ACE also inactivates the vasodilator bradykinin. Together, the two effects result in increased blood pressure. Hence, inhibition of ACE has proven to be an effective strategy for prevention and treatment of hypertension. Several synthetic ACE inhibitory (ACEI) drugs, such as captopril, lisinopril, enalapril, and ramipril, can effectively relieve hypertension; however, some side effects sometimes accompany the use of this category of drug, such as proteinuria, altered sense of taste, allergic skin rashes, cough, and drug fever [9,10]. Therefore, exploration of novel ACE inhibitors from safer natural food sources as alternative medicines is attracting big research interest.

Recently, many ACEI peptides derived from food protein sources have been reported, such as from fermented milk [11], cheddar cheeses [12], hen egg white [13], tuna [14], rice [15], soybean [16], peanut meal [17], skate (*Okamejei kenojei*) skin gelatin [18], flaxseed [19] and pumpkin [20]. These ACEI peptides were commonly released using proteolytic hydrolysis or during fermentation processing. Most ACEI peptides derived from food protein sources show less effectiveness than synthetic ACEI drugs, but they are regarded as safer inhibitors because they are derived from edible food proteins.

Bitter melon (*Momordica charantia*) is widely cultivated in Asian countries and used as a food, especially in Taiwan. Although the bitter melon seeds are usually not consumed directly due to their hard outer shells, they are still a potentially valuable edible protein source for active peptide production due to their high protein content (up to 80.4%) [21]. Some bitter melon seed proteins (BMSPs) have been tested for and showed significant bioactivities, such as anti-HIV [22], anti-cancer [23], and anti-tumor [24]. Thus, the search for novel ACEI peptides from bitter melon seeds is promising, with the potential to increase the utilization and economic value of the seeds. As far as we know, ACEI peptides from bitter melon seeds have not been reported previously. Therefore, the aims of this study were to explore ACEI peptides derived from BMSPs and to evaluate their antihypertensive effects. BMSPs, extracted from dried bitter melon seeds, were digested using various digestion enzymes to hydrolyze them and diverse peptides were produced due to the specificities of proteases [25]. These proteases create numerous bioactive peptides with different lengths and amino acid sequences. We searched for some consensus amino acid sequences with ACE inhibitory properties.

The low molecular weight peptides were collected using 3 kDa molecular weight cut-off ultrafiltration and the peptide mixture was fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). The resulting fractions were screened *in vitro* for ACEI activities. The peptides in the fraction with the best inhibition were sequenced with reference to liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database search [26]. Synthetic peptides were used to confirm the peptide identities characterized by LC-MS/MS and to determine the IC_{50} values of the target peptides. The exact quantities of target peptides in the crude hydrolysates were also determined using multiple reaction monitoring (MRM) mode during LC-MS/MS analysis for the optimization of hydrolysis conditions. Similar to the comprehensive study of ACEI peptides presented by Duan *et al.* [27], in-depth investigations about the mechanism of inhibition of the ACEI peptides were done using Lineweaver–Burk plots and molecular docking calculations for the peptide inhibitors and ACE. Finally, *in vivo* studies of the most active ACEI peptide were also carried out using an animal model of spontaneously hypertensive rats (SHRs) to examine antihypertensive effects.

2. Materials and methods

2.1. Materials and chemical reagents

Bitter melons were collected from Pingtung County, Taiwan, in July 2013. ACE (EC 3.4.15.1) from rabbit lungs, iodoacetamide (IAM), sodium dodecyl sulfate (SDS), hippuryl-L-histidyl-L-leucine (HHL), hippuric acid (HA), trypsin (from bovine pancreas), α-chymotrypsin (from bovine pancreas), pepsin (from porcine gastric mucosa), alcalase (from *Bacillus licheniformis*), and thermolysin (from *Bacillus thermoproteolyticus rokko*) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific Inc. (Rockford, IL, USA). Trifluoroacetic acid (TFA), acetonitrile (ACN), formic acid (FA), boric acid, ammonium bicarbonate (ABC), trichloroacetic acid (TCA), 1,4-dithiothreitol (DTT), and acetone were purchased from J.T. Baker (Phillipsburg, NJ, USA). The synthetic peptides were provided by Vaccine Research and Development Center (NIH, Taiwan). Molecular weight cut-off (MWCO) ultrafiltration membranes (3 kDa) were obtained from Millipore (Bedford, MA, USA). Other chemicals used in this investigation were all of reagent grade. The water used in this study was obtained from a Milli-Q® (Millipore) water purification system (Billerica, MA, USA).

2.2. Extraction of bitter melon seed proteins

Bitter melon seeds were separated from fruit pulp and the seeds were dried in an oven at 50 °C for two days. The grinding of the dried bitter melon seeds was conducted using a grinder (Rong Tsong Precision Technology Co., Taichung, Taiwan), followed by sieving in 100 mesh (Retsch Technology GmbH, Haan, Germany) to obtain homogeneous powders. The powders were re-suspended in phosphate buffered saline (PBS) with ratio 5:1 (*v*/*w*). Cell membrane disruption was carried out using a Branson Digital Sonifier® (Terra Universal Inc., LA, USA), for which the sonicator horn was placed in the center of the solution. The sonication was carried out 4 times, with the amplitude and pulse duration adjusted to 30% and 30 s, respectively. Supernatant and precipitate were separated by centrifugation (14,000 rpm, 5 min, 4 °C) and the supernatant was transferred into fresh tubes. The supernatant was lyophilized and the lyophilized powders were re-suspended in 1% SDS. The solution was centrifuged (14,000 rpm, 5 min) and the supernatant was transferred into a fresh tube. 10% TCA in acetone was added to the supernatant with a ratio of 2:1 (*v*/*v*) and incubated at −20 °C for 30 min. Then the solution was centrifuged at 4 °C for 10 min. The supernatant was removed and the precipitate was lyophilized to yield the protein pellet.

2.3. Enzymatic digestion of BMSPs and the optimization of target peptide preparation

The BMSPs were treated with a single protease, with an enzyme-to-protein ratio of 1:50 (*w*/*w*) using different temperatures which were based on the enzymes' activities: trypsin (37 °C), α-chymotrypsin (37 °C), pepsin (37 °C), alcalase (50 °C), and thermolysin (60 °C). The enzymatic digestions of the BMSPs were kept at pH 8, except for pepsin, which was adjusted to pH 1.5. After incubation for 12 h, the hydrolysis was stopped by centrifugation at low temperature (14,000 rpm, 25 min, 4 °C) in ultra-filtration membrane (3 kDa MWCO). The filtrate (<3 kDa) was lyophilized and kept at −20 °C for further assay or analysis. The optimum conditions for thermolysin hydrolysis of BMSPs were monitored based on various enzyme-to-protein ratios (1/50, 1/100, 1/200, 1/400, and 1/800) (*w*/*w*), hydrolysis times (1, 3, 6, 9, 12, and 15 h) and temperatures of incubation (40, 50, 60, and 70 °C). The optimum condition was defined as the condition which could produce the highest amount of target peptide.