Original Article

Antihypertensive effect of rice protein hydrolysate with *in vitro* angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats

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Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure as well as cardiovascular function. ACE catalyzes the conversion of angiotensin I to vasoconstrictor angiotensin II, and also inactivates the antihypertensive vasodilator bradykinin. Inhibition of ACE mainly results an overall antihypertensive effect. Food protein-derived peptides can have ACE-inhibiting properties and thus may be used as a novel functional food for preventing hypertension as well as for therapeutic purposes. In the present study, rice protein was hydrolyzed by protease Alcalase for 2 h and the resulted hydrolysate was determined for ACE inhibitory activity *in vitro*. The antihypertensive effect of rice protein hydrolysate was also investigated in spontaneously hypertensive rats (SHR). The Alcalase-generated hydrolysate showed strong *in vitro* ACE inhibitory activity with the IC_{50} value of 0.14 mg/ml. A significant decrease in systolic blood pressure in spontaneously hypertensive rats was observed following single oral administration of this hydrolysate at a dose of 600 mg/kg of body weight. A potent ACE inhibitory peptide with the amino acid sequence of Thr-Gln-Val-Tyr (IC_{50}, 18.2 μM) was isolated and identified from the hydrolysate. Single oral administration of Thr-Gln-Val-Tyr at a dose of 30 mg/kg of body weight also significantly decreased blood pressure in SHR. These results suggest that i*n vitro* ACE inhibitory activity and *in vivo* antihypertensive activity could be generated from rice protein by enzymatic hydrolysis. The rice protein hydrolysate prepared with Alcalase might be utilized to develop physiologically functional food with antihypertensive activity.

Key Words: angiotensin I-converting enzyme, rice protein, antihypertensive effect, spontaneously hypertensive rats, Alcalase

Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxpeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as cardiovascular function. ACE converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II, and also inactivates vasodilator, bradykinin. [1] Thus, inhibition of ACE results in a decrease in blood pressure. Many potent synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and ramipril have been widely used in the clinical treatment of hypertension and heart failure in humans. However, synthetic ACE inhibitors can have side effects including cough, taste disturbances and skin rashes. [2] Therefore, the search for diet-related preventive measures for hypertension is obviously of interest within the scope of functional foods. ACE inhibitory peptides derived from food proteins are suitable candidates for such products. Many ACE inhibitory peptides have recently been discovered from enzymatic hydrolysates of different food proteins. It has been well demonstrated that these peptides with *in vitro* ACE inhibitory activities show *in vivo* inhibitory properties on ACE and antihypertensive effects without side effects in spontaneously hypertensive rats (SHR) and hypertensive humans. [3]

Rice (*Oryza sativa* L.) is the main staple food for more than half of the world's population, mostly in Asian countries. [4] The component of protein in rice, at 7–9% by weight, is relatively low, but it is a major source of protein for these rice-consuming people. Rice protein possesses unique nutritional properties with being colourless, hypoallergenic, rich in essential amino acids, and has a bland taste. [5] Besides being as a staple diet for human consumption, rice is also an important starting material for starch, dextrin, grape sugar and syrup manufacturing.

A mass of rice residues are produced during processing of these products. The rice residue contains up to 50% protein by dry weight and is considered as low –cost industrial co-product. [6] However, the rice residual proteins have poor solubility in water due to the presence of a substantial amount of insoluble glutelin accounting for more than 80% of the total residual proteins, [7] which makes it under-used and under-valued through years.

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In the present study, rice protein hydrolysate with *in vitro* ACE inhibitory activity was produced by enzymatic hydrolysis with commercially available protease Alcalase. We also investigated the antihypertensive effect of the hydrolydate using SHR. A potent ACE inhibitory tetrapeptide with antihypertensive activity *in vivo* was isolated and identified from the hydrolysate.

Materials and methods

Materials

Long rice with protein content of 8.8% was obtained from local market. ACE (from rabbit lung; 3.4units/mg of protein), pepsin (1:60,000, 3400 U/mg of solid, from porcine stomach mucosa) and hippuryl-L-histidyl-L-leucine (Hip-His-Leu) were purchased from Sigma. Alcalase 2.4L (liquid, 2.4 AU/g) was kindly provided by Novo Nordisk (Bagsvaerd, Denmark). Alcalase 2.4 L is a food-grade endoprotease from *Bacillus licheniformis* and the main enzyme component is the serine protease subtilisin A (EC3.4.21.62). Corolase PP® (pancreatin, from bovine pancreas) was purchased from Röhm (Darmstadt, Germany). Thr-Gln-Val-Tyr, the ACE inhibitor identified from the rice protein hydrolysate, was synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystems) followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. All other chemical reagents were of analytical grade unless otherwise specified.

Preparation of rice protein isolates

Rice seeds were ground to pass 80-mesh screen. Protein isolates were prepared from rice flour by extraction with 0.085 M NaOH (1:12 w/v flour:water ratio) for 2 h at room temperature. The suspension thus obtained was centrifuged at 3,000×g for 20 min. The supernatant was removed and adjusted to pH 5.5 with 2N HCl. The protein precipitate formed was separated by centrifuged at 3,000×g for 20 min. The precipitate was washed twice with distilled water and then lyophilized.

Preparation of protein hydrolysate

Alcalase and Na_2SO_3 were added to the rice protein isolates solution (4%, w/v) at 20μl/g rice protein and 0.2mM/g rice protein, respectively. Hydrolysis was performed using the pH-stat method [8] for 2 h at 55 ^oC and pH 8.0. The enzymatic hydrolysis was terminated by heating for 10 min in a boiling water bath. The protein hydrolysate was centrifuged at 10000×g for 20 min, and the resulting supernatant was desalinized by ion exchange resin and then lyophilized. The peptide content was measured by UV absorbance difference at 215 and 225 nm. [9]

Assay for ACE inhibitory activity

ACE inhibitory activity was measured by our previously described method. [10] The IC_{50} value was defined as the concentration of peptide in mg /ml required to inhibit 50% of the ACE activity under the assayed conditions, and was determined by regression analysis of ACE inhibition (%) versus log (peptide concentration, mg/ml).

Digest test

Fifty milligrams of hydrolysate were individually incubated in 5 ml pepsin solution (0.05 mg/ml, pH 2.0) for 3 h, or 5 ml pancreatin solution (0.05 mg/ml, pH 8.0) for 4 h. at 37 ℃. In successive digestion test with pancreatin after pepsin treatment, the pepsin solution was heated for 5 min in boiling water and adjusted to pH 8.0, pancreatin was then added to the solution, followed by incubation for 4 h at 37 ℃. The reaction solutions were heated for 5 min in boiling water to terminate the reaction and then centrifuged at 10,000×g for 10 min, the supernatants were used for measurement of ACE inhibitory activity.

Measurement of blood pressure

Twenty-four male SHR (12 weeks old, weighing 305.86±5.22 g) with a systolic blood pressure (SBP)>180 mmHg were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). The rats were randomly divided into 4 groups with 6 rats each. Samples were dissolved in 1.5 ml distilled water and orally administered to SHR by gastric intubation at a dose of 600 mg/kg for rice protein isolates and hydrolysate, and 30 mg/kg of body weight for synthetic peptide Thr-Gln-Val-Tyr, respectively. Control rats were given the same volume of distilled water. SBP was measured before and 2, 4, 6, 8 and 12 h after administration. The SBP measurement was performed by the tail-pulse pick up method using a RBP-1B blood pressure meter after warming the rats in a warm holder kept at 37-39 ℃ for 10 min. This animal experiment was carried out at the Second Medical University of Shanghai (SMUS) according to the guidelines for the animal experimentation of SMUS.

Purification of ACE inhibitory peptide

The lyophilized hydrolysate was loaded onto a Sephadex G-15 column (1.8×60 cm) equilibrated with 20 mM sodium acetate-acetic acid buffer solution (pH 4.0) and eluted with the same buffer solution at a flow rate of 0.4 ml/min. The elution was monitored at 220 nm. The fraction with the highest ACE inhibitory from Sephadex G-15 column was further purified by reverse-phase high performance liquid chromatography (RP-HPLC) with Sephasil Peptide C18 ST 4.6/250 column (4.6×250 mm, Amersham Pharmacia Biotech, Sweden) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) from 0% to 60% over 60 min at a flow rate of 1 ml/min. The elution was monitored at 220 nm. The strongest active peak was further applied onto the Sephasil Peptide C2/C18 ST 4.6/250 column and eluted using a linear gradient of acetonitrile in 0.1% TFA from 10% to 30% over 40 min at a flow rate of 1 ml/min.

Identification of ACE inhibitory peptide

The amino acid composition of purified peptide showing potent ACE inhibitory activity was determined by precolumn derivatization with O-phthalaldehyde on the automatic amino acid analyzer (Agilent HP1100, Agilent Co., USA) after hydrolysis for 24 h in 6 N HCl at 110℃ under vacuum. The sequence of the purified ACE inhibitory peptide was analyzed by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS). All the mass spectra and tandem mass spectra were acquired using manual method by the ABI 4700 TOF-TOF Proteomics Analyzer instrument

(Applied Biosystems, Framingham, MA, USA) in the reflectron and positive ionization mode.

Statistical analysis

All results are expressed as means ± S.E.M. Statistical comparisons of the results between two groups were performed by Student's *t*-test. Values of $p<0.05$ were considered significant.

Results

ACE inhibitory activity of rice protein hydrolysate

Rice protein isolates were hydrolyzed with Alcalase for 2 h and the generated hydrolysate was subjected to assay for ACE inhibitory activity. The non-hydrolyzed rice protein isolates showed no inhibitory activity on ACE. ACE inhibitory activity was generated from the rice protein after enzymatic hydrolysis. The hydrolysate showed potent ACE inhibitory activity after 2 h incubation with the IC_{50} value being 0.14 mg protein/ml. This result suggested that peptides released from natural rice protein by enzymatic hydrolysis were responsible for ACE inhibition. Hydrolysis is necessary in order to release ACE inhibitory peptides from an inactive form within the sequence of rice protein. On the other hand, the hydrolysate obtained has good solubility in water.

Digestion stability and antihypertensive effect of hydrolysate in SHR

The inhibitory potencies of the peptides on ACE activity did not always correlate with their *in vivo* antihypertensive effects as showed in some studies. [3,11,12,13] In order to exert an antihypertensive effect *in vivo*, the ACE inhibitory peptides must be absorbed in their intact form from intestine and further be resistant to plasma peptidases degradation to reach their target sites after oral administration. To investigate the resistance of rice protein hydrolysate to digestion by gastrointestinal proteases, the hydrolysate was further treated by digestive proteases. Digestion stability was evaluated by the change in IC_{50} values of hydrolysate before and after treatment with gastrointestinal proteases by simulation of *in vivo* digestion. As shown in Table 1, the ACE inhibitory activity of hydrolysate was slightly decreased after treatment with gastrointestinal proteases, suggesting that the hydrolysate still retained its ACE inhibitory activity after digestion with gastrointestinal enzymes and thus may exert antihypertensive effect after oral administration.

To further validate the *in vivo* hypotensive activity of the hydrolysate, SHR were administrated orally with rice protein hydrolysate prepared with Alcalse at a dose of 600 mg/kg of body weight. The antihypertensive effect was evaluated by measuring changes in SBP after single oral administration. Fig 1 shows the time-course changes in SBP after oral administration of non-hydrolyzed rice protein, rice protein hydrolysate, synthetic peptide Thr-Gln-Val-Tyr and distilled water. Before administration of the different samples, the mean SBP of SHR was 187.9±8.7 mmHg. The oral administration of non-hydrolyzed rice protein and distilled water showed no hypotensive effect. However, rice protein hydrolysate at a dose of 600 mg/kg caused a significant decrease in SBP in SHR. Maximum SBP reduction of 25.6 mmHg was observed 6 h after administration. The blood pressure-lowering effect continued for at least 8 h and the blood pressure of SHR returned to initial levels at 12 h after administration. The heart rate in SHR was also measured after single oral administration in order to check the effect of the administration of the hydrolysate on the physical condition. No significant changes in the heart rate of SHR were observed in all groups at 2, 4, 6, 8 and 12 h after oral administration (data not shown), suggesting that the administration of the hydrolysate did not have a bad effect on the circulatory system of SHR.

Table 1. Effects of gastrointestinal proteases treatments on the ACE inhibitory activity of rice protein hydrolysate

Digestive protease	IC_{50} (mg protein/ml)
None	0.14
Pepsin	0.15
Pancreatin	0.20
Pepsin ⟶ Pancreatin	0.18

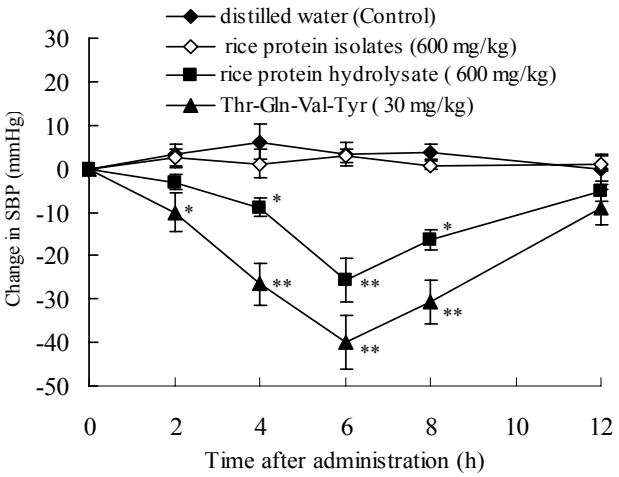

Figure 1. Changes in systolic blood pressure (SBP) of SHR after single oral administration of different products. Significant difference from control: $^{*}p<0.05$; $^{**}p<0.01$.

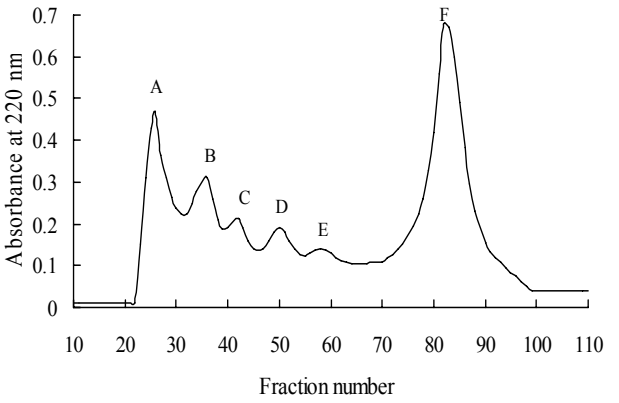

Figure 2. Gel filtration chromatography profile of the Alcalase hydrolysates of rice protein on Sephadex G-15 column. Separation was performed at a flow rate of 0.4 ml/min with 20 mM sodium acetate-acetic acid buffer solution (pH 4.0). Elution profile was monitored at 220 nm.

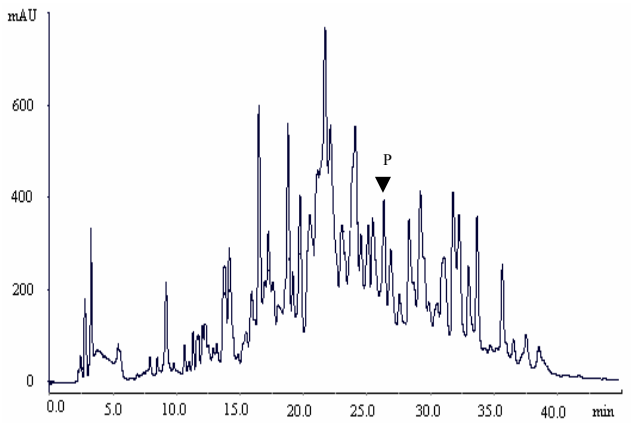

Figure 3. Reverse-phase HPLC on a Sephasil Peptide C18 ST 4.6/250 column of fraction D from Sephadex G-15 column. Elution profile was monitored at 220 nm. Peak P showed the strongest ACE inhibitory activity.

Purification of ACE inhibitory peptide

To identify the ACE inhibitory peptide contained in the hydrolysate which had demonstrated ACE inhibitory activity *in vitro* and antihypertensive activity *in vivo* as described above, the hydrolysate was initially separated using Sephadex G-15 gel filtration chromatography. The hydrolysate was fractionated into six individual fractions (fraction A-F, Fig 2) by Sephadex G-15 column, among which fraction D possessed the strongest inhibitory activity against ACE. Fraction D was further purified by RP-HPLC on a Sephasil Peptide C18 column. Fractions eluted from 16% to 38% acetonitrile showed various levels of ACE inhibitory activity (Fig 3). As the strongest activity was observed in peak designated as P, this peak was purified to homogeneity by a second RP-HPLC using Sephasil Peptide C2/C18 column (data not shown). The purified peak was identified by amino acid composition analysis and MALDI-TOF MS/MS as Thr-Gln-Val-Tyr with the IC_{50} value of 18.2 μM.

The antihypertensive activity of Thr-Gln-Val-Tyr was also tested following oral administration to SHR. As shown in Fig. 1, oral administration of Thr-Gln-Val-Tyr at a dosage of 30 mg/ml significantly decreased blood pressure by approximately 40 mmHg 6 h after oral administration (p<0.01).

Discussion

Besides supplying amino acids and energy that are essential for growth and maintenance, food proteins can act as an important source of biologically active peptides with antihypertensive, opioid, immunomodulating, antioxidative, antimicrobial, antithrombotic, antiamnestic, hypocholesterolemic and other activities. [3,14,15] These peptides are inactive within the sequence of parent proteins, but they can be released by enzymatic proteolysis *in vivo* or *in vitro*, for example during gastrointestinal digestion or during food processing. Recent studies have shown that rice protein exhibited antidiabetic, [16]antitumor, [17] hypocholesterolemic, [18] and immunomodulatory activities. [19] In the present study, we have demonstrated that enzymaticly hydrolyzed rice protein by commercially available protease Alcalase exhibited potent *in vitro* ACE inhibitory activity and the hydrolysate exerted antihypertensive effect in SHR after single oral administration.

The SHR strain, in which the development of hypertension is very similar to that in human, has extensively been used to test the acute and/or long-term antihypertensive effects of functional food products and bioactive peptides derived from food proteins. [13, 20-28] In clinical trials, Hata et al reported that sour milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* reduced systolic and diastolic blood pressure in an 8-week intervention in a placebo-controlled study of hypertensive patients. [29] *L. helveticus* LBK-16H fermented milk product containing ACE inhibitory peptides also showed short- and long-term blood pressure-lowing effects in hypertensive patients. [30,31] In a randomized double-blind placebo-controlled study, Kawasaki et al found that a vegetable drink containing sardine protein hydrolysates with *in vitro* ACE inhibitory activity exhibited the antihypertensive effect in only the subjects with mild hypertension or high-normal blood pressure, and no adverse effects were observed in either hypertensive or normotensive subjects. [32]

Similar results were also observed with respect to thermolysin digest of dried bonito called "Katsuobushi oligopeptide", which contains ACE inhibitory peptide Leu-Lys-Pro-Asn-Met. [33] This product has been officially approved as Foods for Specified Health Use by the Ministry of Health and Welfare in Japan. In this study, a single oral administration of Alcalase hydrolysate rice protein at a dosage of 600 mg/kg to SHR caused a prolonged blood-lowing effect up to 8 h with a maximum reduction of 25.6 mmHg 6 h after administration, whereas the non-hydrolyzed rice protein showed no antihypertensive activity. Meanwhile, Thr-Gln-Val-Tyr contained in the hydrolysate also significantly lowered blood pressure of SHR after a single oral administration at 30 mg/ml with the maximum effect occurring at the same time as that of hydrolysate. The substantial antihypertensive activity of Thr-Gln-Val-Tyr presented in the hydrolysate could in part account for the potent blood pressure-lowering effect of rice protein hydrolysate. Both hydrolysate and Thr-Gln-Val-Tyr showed no effect on the heat rate of SHR. It was found that ACE inhibitory peptides present in the rice protein hydrolysate are relatively resistant to digestion by gastrointestinal enzymes, as ACE inhibitory activity of the hydrolysate was changed slightly after treatment with digestive enzymes (Table 1). Alcalase hydrolysate of rice protein, therefore, might be used as a physiologically functional food with potential benefits in the prevention and/or treatment of hypertension. However, in this study, we only conducted the acute blood pressure-lowering effects of rice protein hydrolysate and ACE inhibitory peptide therein by oral administration of single dosage.

Further investigations are obviously necessary to evaluate the dose-response effects of the long-term intake of these products on the development of hypertension of model animals. Moreover, before routine clinical use of rice protein hydrolysate, it would be necessary to carry out clinical studies to demonstrate its long-term antihypertensive efficiency in humans.

Conclusions

In conclusion, we have demonstrated that rice protein hydrolysate prepared with Alcalase inhibited ACE activity *in vitro* and exerted antihypertensive effect *in vivo* in

SHR after single oral administration. A potent ACE inhibitory tetrapeptide Thr-Gln-Val-Tyr was identified from the hydrolysate and also showed blood pressure-lowering activity when orally administrated to SHR. Alcalase hydrolysate of rice protein could be used as a physiologically functional food with antihypertensive activity. However, extended clinical trials in human volunteers are necessary to further evaluate their long-term efficacy and safety before considering the exploitation of this product in physiologically functional foods for preventing hypertension as well as for therapeutic purposes.

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