ANNUAL REPORT
COMPREHENSIVE RESEARCH ON RICE
January 1, 2009– December 31, 2009

PROJECT TITLE:

RICE UTILIZATION AND PRODUCT DEVELOPMENT
-Development of Functional Polypeptides from Rice Protein

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OBJECTIVES AND EXPERIMENTS CONDUCTED TO ACCOMPLISH OBJECTIVES

Broken rice and rice bran are produced in large quantities as by-products from rice processing. Currently they have low values and have not been well utilized. Both of them contain high protein contents and can be used as raw materials for producing high-value functional polypeptides which have great market potential as functional food ingredients and nutritional supplements. The food protein-based antihypertensive peptides have no or fewer side effects and are safer compared to conventional antihypertensive drugs which are angiotension converting enzyme (ACE) inhibitors. The development of new processing methods for producing these functional (antihypertensive) polypeptides from rice protein is curial. Consequently, in this research ultrasonic technology is explored as a new processing method for processing functional polypeptides from rice protein. Ultrasonic technology has many advantages in promoting mass transfer and enzyme modification over conventional extraction and enzymatic hydrolysis methods. It can be used for both protein extraction and enzyme hydrolysis in the production of functional polypeptides for increased functionality and reduced production time.

Objectives

The objectives of this past year research were as follows

1. Optimize the processing conditions of ultrasonic assisted extraction and pretreatment.
2. Study the antihypertensive properties of different fractions of peptides separated by using ultrafiltration.
3. Investigate the in vitro stability of the antihypertensive polypeptides and the antihypertensive effect on Spontaneous Hypertension Rats (SHR).

Technical approaches

Optimization of processing conditions of ultrasonic assisted extraction and pretreatment

Based on antihypertensive activity (judged by the inhibitory rate of angiotensin ?-converting enzyme, ACE) of rice protein's hydrolysate, Alcalase was chosen from four proteases to digest rice protein for producing antihypertensive polypeptides product. The optimal digestion conditions of Alcalase were determined according to orthogonal test at the following conditions: substrate 4% (m/v), Alcalase 7.5% (E/S), digestion temperature 50^{o}C, and pH 9.5.

In order to accelerate rice protein digestion by protease, and to enhance the bio-activity of polypeptides, the ultrasonic pretreatment experiments of rice protein were carried out and the pretreated protein was subsequently digested by Alcalase according to the above

conditions. In single-factor experiments, the ultrasonic power (P), the ratio of ultrasound emission/intermission time (E/I), the circulation stirring rate (SR), and ultrasonic emission total time (Tt) were explored based on the hydrolysate's antihypertensive activity. The optimal pretreatment conditions for P, Tt, E/I and SR were determined as, 500 w (250 w/L), 10 min, 1:2 and 30 r/s, respectively. The ultrasonic pretreatment led to significant increase of inhibitory rate of hydrolysates to ACE. The value of the inhibitory rate of hydrolysates to ACE increased up to 92.8% from 69.8% of the control without ultrasonic pretreatment.

Separation of polypeptides by using ultrafiltration

Filtration by three-column filtering centrifuge (Huada centrifuge Co. Ltd, Zhangjiagang, China) was conducted and the enzymolysis product was ultrafiltered with 10k, 5k, and 3k Dalton membranes in sequence (Fig. 1) by a Millipore ultrafiltration system (Pellicon 2 Ultrafiltration, Millipore Co. Ltd, USA). In the whole ultrafiltration process, the filtration temperature was all maintained at 30°C. After the ultrafiltration, polypeptides were divided to four parts, each molecular weight was MW>10k, <10k and >5k, <5k and >3k, and<3k, respectively. And IC_{50} value of each part was detected to investigate the in vitro antihypertensive activity of rice polypeptides with different MW.

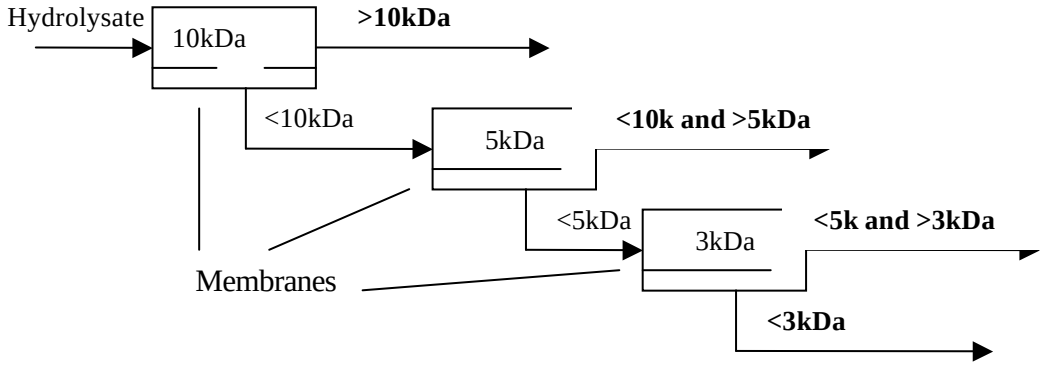

Fig.1 Diagram of the separation of polypeptides by using ultrafiltration

In vitro stability of the antihypertensive polypeptides and the antihypertensive effect on SHR

In order to determine in vitro stability, the activity of preserved rice antihypertensive polypeptides was evaluated at different temperatures and pH conditions. Rice peptides within 3k of MW were stored at different temperatures (4, 37, and 90°C and at pH 7.0) or different pH (2, 7, and 12, and at 37 °C) conditions to carry out in vitro stability experiment.

In order to determine in vivo antihypertensive effects, spontaneously hypertensive rats (SHRs) were fed with rice polypeptides. Rice polypeptides of MW<3k were fed to SHRs (6 in a group). The SHRs were given different doses of rice polypeptides by a single gastric intubation each. Then the systolic blood pressures of rats were measured to investigate any decrease which indicates an effect on depressing blood pressure in vivo. Another group with 5mg/kg body weight which was Captopril fed was included in this research. And distilled water was used as control.

SUMMARY OF 2009 RESEARCH RESULTS (MAJOR ACCOMPLISHMENTS)

Optimization of processing conditions of ultrasonic assisted extraction and pretreatment

Selection of protease for polypeptides production

The enzymatic hydrolysis method which was employed in this research is described stepwise as follows. The rice protein was resolved in distilled water, and hydrolyzed by four proteases according to recommended condition by enzyme manufacturers (Table 1), respectively. Four proteases (Table 1) were tested to elucidate the digestion of rice protein for antihypertensive polypeptides product, and their enzymatic hydrolysis time were 40, 30, 60, 75 min, respectively. The digested protein solutions were boiled for 10 min to inactivate the enzyme, and then filtered. The supernatant contains majority of ACE-inhibitory polypeptides. ACE-inhibitory activity of the supernatant (diluted to 1:20 v/v) obtained with 75 min enzymatic hydrolysis was determined based on the method by Cushman and Cheung, with slight modifications. The results are shown in Figure 2.

Table 1 Proteases and their recommended hydrolysis conditions by manufacturers

Protease	Manufacturer	Recommending Enzymatic Hydrolysis Condition
Alcalase	Novo Enzyme Co. Ltd, Danmark	50? , pH9.0
Neutrase	Xingda Enzyme Co. Ltd, Wuxi, China	50? , pH7.0
Papain	Novo Enzyme Co. Ltd, Danmark	60? , pH6.0
Trypsin	Novo Enzyme Co. Ltd, Danmark	37? , pH8.0

Fig. 2 ACE inhibitory rate of hydrolysates prepared by different proteases

It was found that hydrolysate by Alcalase has the highest ACE inhibitory activity among the four proteases and we chose it for the subsequent studies.

Single-factor tests were investigated to explore the effects of substrate content, E/S, and digestion pH value on hydrolysate of rice protein by Alcalase. The hydrolysis time of each test was 40 min. The results are shown in Fig. 3 to 5.

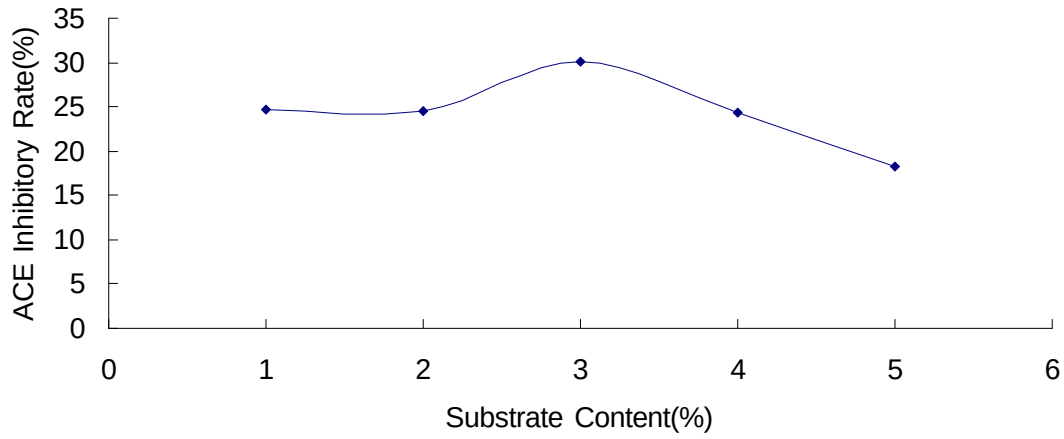

Fig. 3 ACE inhibitory rate of hydrolysates digested for different rice protein contents

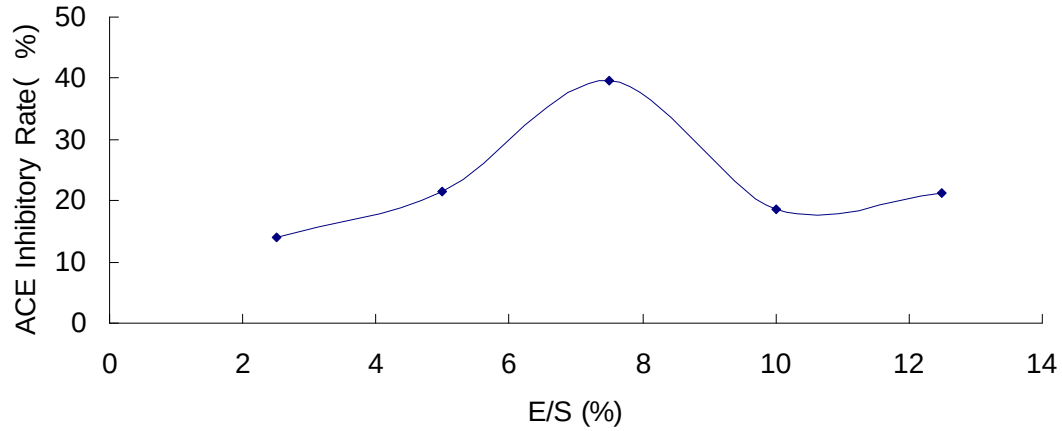

Fig. 4 ACE inhibitory rate of hydrolysates digested by different contents of Alcalase (Enzyme/Substrate, w/w)

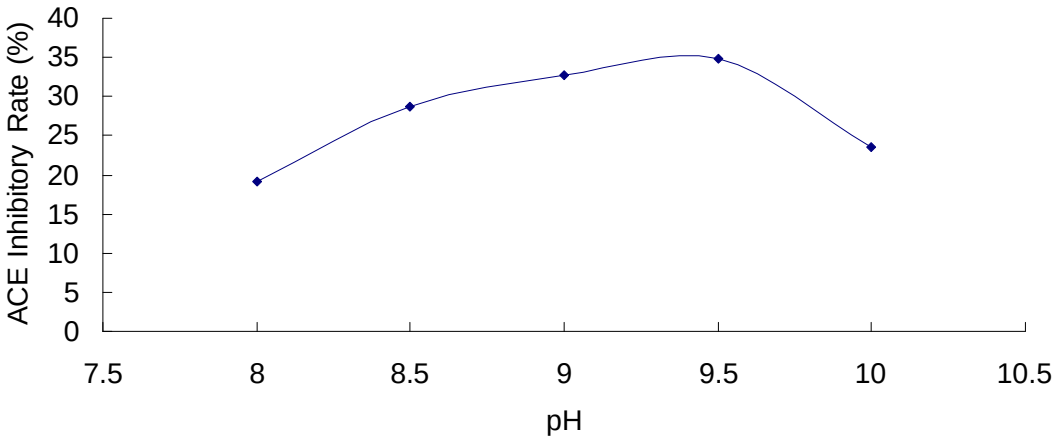

Fig. 5 ACE inhibitory rate of hydrolysates digested at different pH values

The above results of single-factor tests indicated that the hydrolysates of rice protein had the relatively high ACE-inhibitory rates at conditions of 3% rice protein, by 7.5% (E/S) Alcalase, and at about pH 9.5, respectively.

Based on the single-factor tests, an orthogonal test of the rice protein digestion for antihypertensive polypeptides was plotted and carried out according to Table 2. The range analysis of the orthogonal test was run and the results are shown in Table 2.

Table 2 Experimental design and results of orthogonal test for rice protein digestion

Tests	Substrate(%)	E/S(%)	pH	Error	ACE Inhibitory Rate (%)
1	1(2)	1(5)	1(8.5)	1	48.6
2	1	2(7.5)	2(9)	2	57.1
3	1	3(10)	3(9.5)	3	54.8
4	2(3)	1	2	3	55.2
5	2	2	3	1	74
6	2	3	1	2	59.3
7	3(4)	1	3	2	68.2
8	3	2	1	3	66.3
9	3	3	2	1	60
K1	53.5	57.333	58.067	60.867	Σ=543.5
K2	62.833	65.8	57.433	61.533	
K3	64.833	58.033	65.667	58.767	
Kj	11.833	8.467	8.234	2.766	

The range analysis determined the affecting sequence of the factors as follows: substrate content > E/S > pH, and the optimal digestion conditions for obtaining the highest ACE inhibitory rate were the substrate content of 4%, E/S of 7.5%, and pH of 9.5 under 40 min of digestion.

The variance analysis results are shown in Table 3. The substrate, E/S and pH all significantly affected polypeptides activity of rice protein.

Table 3 Variance analysis of orthogonal test for rice protein digestion[#]

Factor	DEVSQ	DOF	F Ratio	F Critical Value	Significance
Substrate	219.556	2	17.552	9	*
E/S	132.496	2	10.592	9	*
pH	125.949	2	10.069	9	*
Error	12.51	2			

[#] (α<0.10)

Ultrasonic pretreatment of rice protein for Antihypertensive polypeptides production

Rice protein solution (200mL) was ultrasonically pre-irradiated using a sonicator (20kHz, ShangJia Bio-technological Ltd. Co., Jiangsu, P.R.C.). The pre-irradiated protein solution was then enzymatically hydrolyzed according to the method described in the previous section (*Protease selection*) which involved inactivation of the enzyme by boiling for 10 min and centrifugation of the supernatant. The ACE-inhibitory activities of hydrolysates were measured after the hydrolysis reaction.

Single-factor tests of ultrasonic pretreatment were investigated, and the results are presented in Fig. 6 to 9. The results of single-factor tests indicated that relatively higher ACE inhibitory rates were found in the hydrolysates of rice protein pretreated at ultrasonic power of 400W, pretreatment time of 15 min, and stirring rate of 30 r/min.

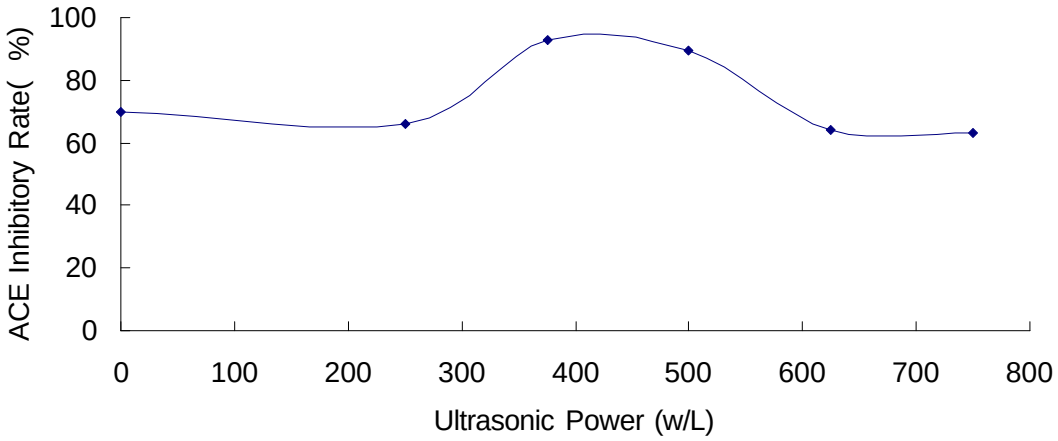

Fig. 6 Effect of ultrasonic pretreatment power on ACE inhibitory rate of hydrolysates

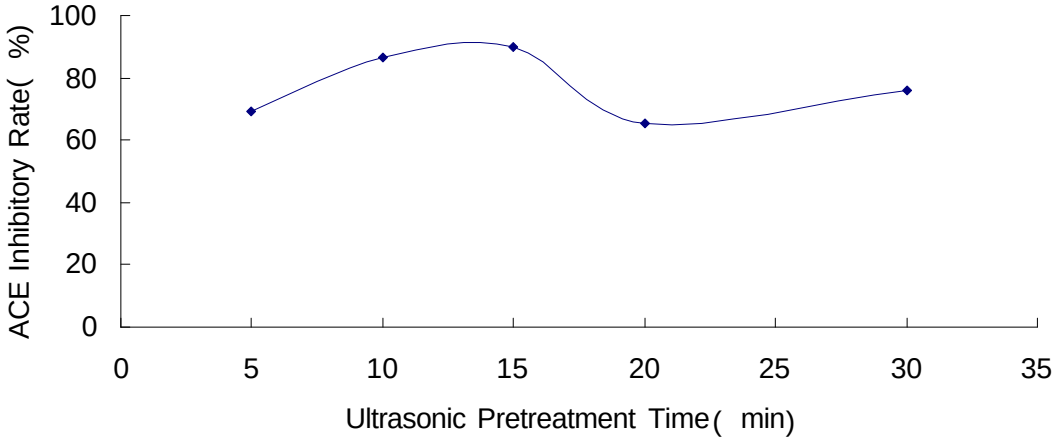

Fig. 7 Effect of ultrasonic pretreatment time on ACE inhibitory rate of hydrolysates

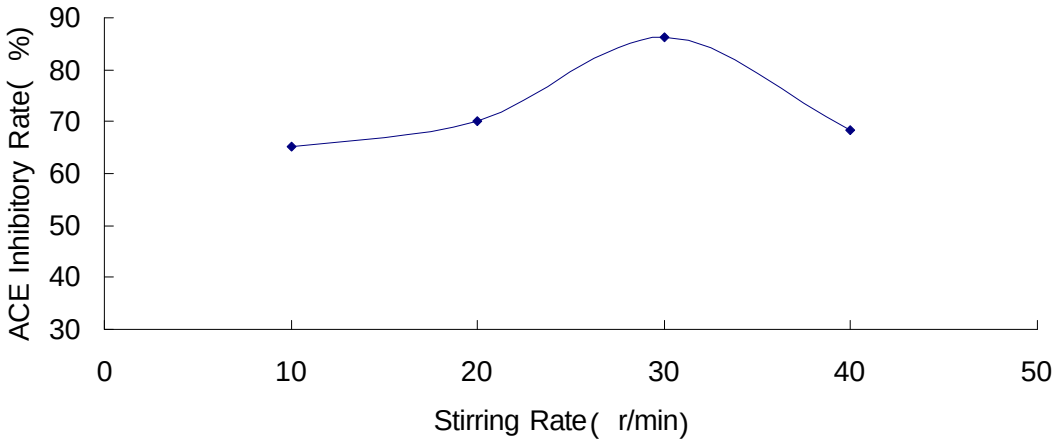

Fig. 8 Effect of ultrasonic pretreatment stirring rate on ACE inhibitory rate of hydrolysates

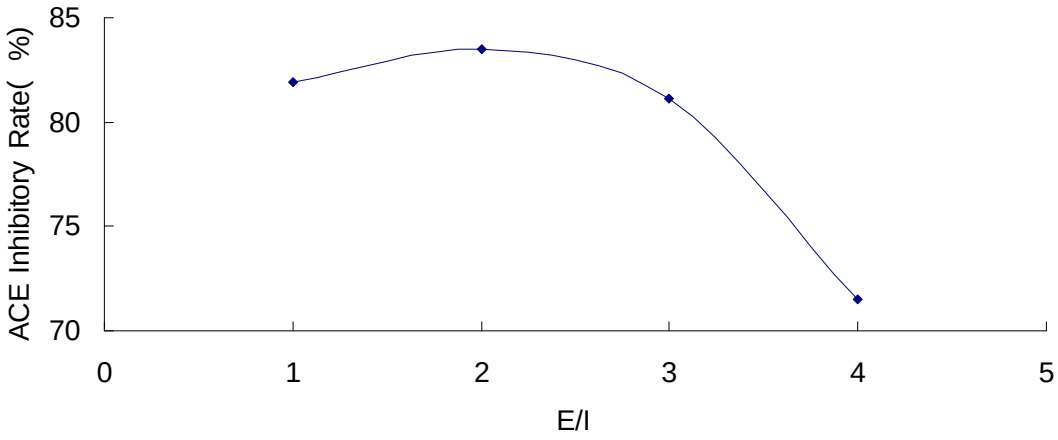

Fig. 9 Effect of ultrasonic pretreatment emission/intermissio n ratio (E/I, s/s) on ACE inhibitory rate of hydrolysates

Based on the single-factor tests, an orthogonal test of the rice protein ultrasonic pretreatment for antihyper tensive polypeptides was designed and carried out according to Table 4. Range analysis of the orthogonal test was run and the results are shown in Table 4. The range analysis determined the affecting sequence of the factors as follows: E/I > Time > Power > Stirring rate. The variance analysis results are shown in Table 5. It was shown that E/I and Time of ultrasonic pretreatment significantly affected polypeptides activity of rice protein.

Table 4 Experimental design and results of orthogonal test for rice protein ultrasonic pretreatment

Tests	Power(w)	Time(min)	E/I(s/s)	Stirring rate(r/s)	ACE Inhibitory Rate (%)
1	1(500)	1(5)	1(1:2)	1(20)	85
2	1	2(10)	2(1:1)	2(30)	69.5
3	1	3(15)	3(2:1)	3(40)	54.8
4	2(750)	1	3	2	69.8
5	2	2	1	3	85.7
6	2	3	2	1	43.3
7	3(1000)	1	2	3	46.1
8	3	2	3	1	54.2
9	3	3	1	2	56.2
K1	69.767	66.967	75.633	60.833	Σ=564.6
K2	66.267	69.8	52.967	65.167	
K3	52.167	51.433	59.6	62.2	
Kj	17.6	18.367	22.666	4.334	

Table 5 Variance analysis of orthogonal test for rice protein digestion[#]

Factor	DEVSQ	DOF	F Ratio	F Critical Value	Significance
Power	520.82	2	17.687	19	
Time	586.647	2	19.922	19	*
E/I	814.847	2	27.672	19	*
Stirring Rate	29.447	2	1	19	
Error	29.45	2			

[#](α<0.05)

The optimal digestion conditions were determined as follows: ultrasonic power (P) of 500W, ultrasonic emission total time (Tt) of 10 min, emission/intermission ratio of 1:2, and stirring rate of 30r/min. This condition is not in the orthogonal test. Rice protein was pretreated according to the optimal condition, then hydrolyzed by Alcalase and compared with control (without ultrasonic pretreatment). The ACE-inhibitory rate of polypeptides produced under the optimal condition is 92.8%, whereas that of the control is 69.8%.
The above experimental research results indicated that ultrasonic pretreatment of rice protein significantly increased the hydrolysate's ACE-inhibitory activity.

Separation of polypeptides by using ultrafiltration

The enzymolysis product was ultrafiltered by 10k, 5k, and 3k Dalton membranes in sequence (filtered by three-column filtering centrifuge). The IC_{50} (content of polypeptides at which 50% ACE activity is inhibited) of each part was 2.87mg/ml (the part with MW>10k), 1.71mg/mL (<10k and >5k), 1.588mg/ml (<5k and >3k), 1.515 mg/ml (<3k),

respectively. These results indicated that the rice polypeptides with the highest ACE-inhibitory activity are those with the MW of lower than 3k Dalton. The distribution of ACE-inhibitory activity of different MW polypeptides is shown in Fig. 10. The result shows that the rice polypeptides parts in which MW were under 10k Dalton had high ACE-inhibitory activity, and the parts in which MW<3k had the highest activity in vitro. Generally, the lower the IC_{50} is corresponding to the higher the polypeptides' ACE-inhibitory activity.

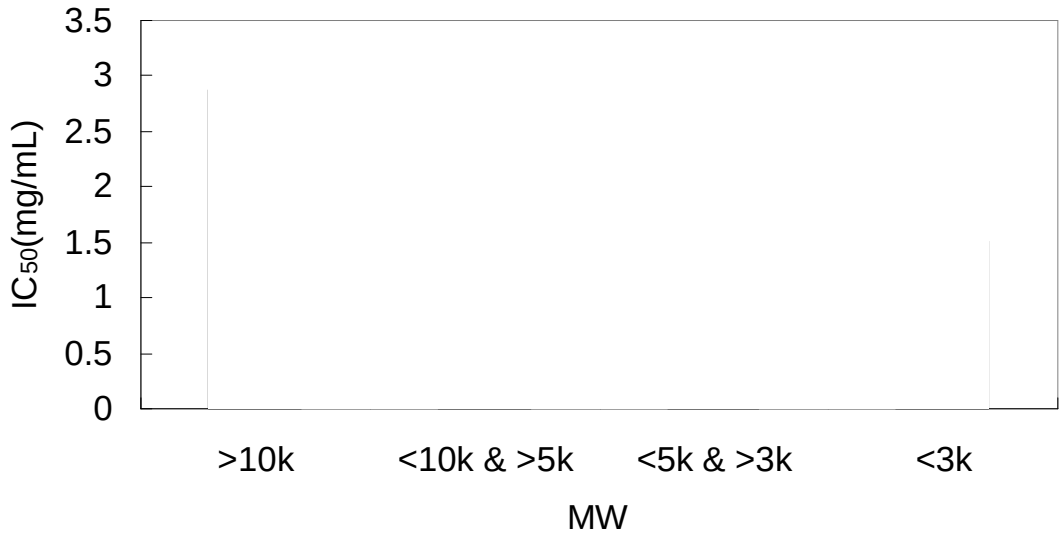

Fig. 10 IC_{50} values of different MW rice polypeptides

In vitro stability of antihypertensive polypeptides and antihypertensive effect on SHR

Rice peptides within 3k of MW were stored at different temperatures (4, 37, and 90^{o}C) and at pH 7.0 or different pH (2, 7, and 12) and at 37 ^{o}C in order to carry out in vitro stability experiment, respectively. Fig. 11 shows ACE-inhibitory activities of rice peptides at different temperatures of storage.

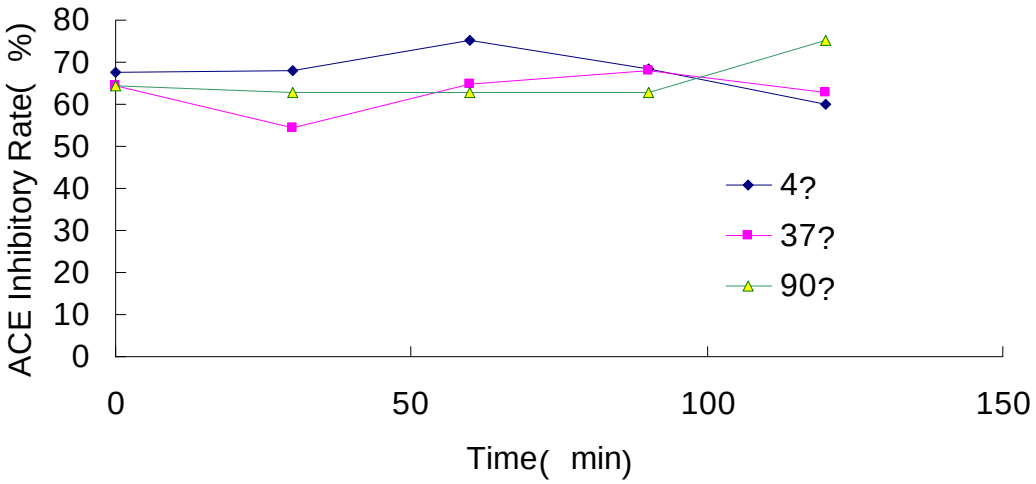

Fig. 11 Effect of storage temperature on ACE inhibitory rate of rice peptides

From Fig. 11, it was found that ACE-inhibitory rate of three samples stored at 4, 37, and 90°C had no remarkable change. Therefore it could be concluded that the antihypertensive activity of rice peptides was stable at the different temperatures of storage in this experiment.

ACE-inhibitory activities of rice peptides during storage at different pH conditions are presented in Fig. 12. Rice peptides samples stored at different pH conditions (2, 7, and 12) did not show significant change in ACE-inhibitory rate which demonstrated their high stability at different pH value condition.

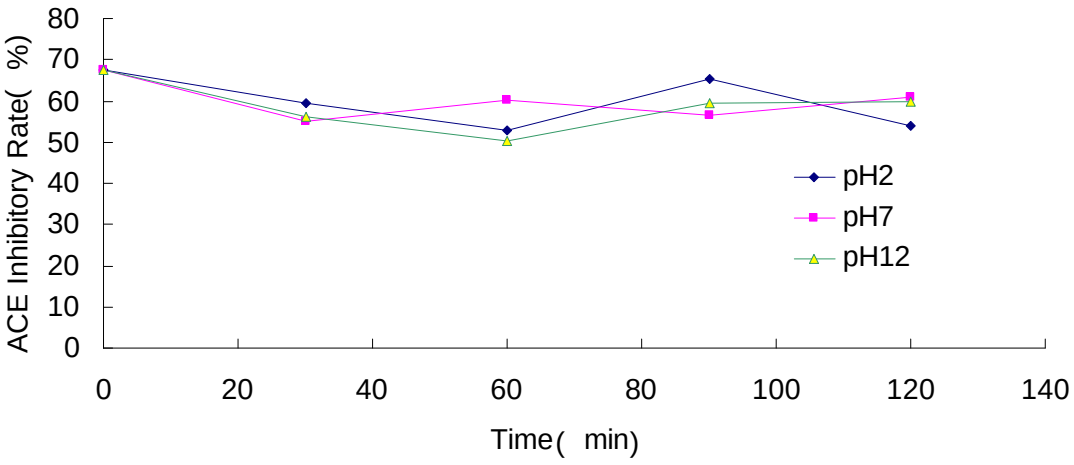

Fig. 12 Effect of storage pH on ACE inhibitory rate of rice peptides

Rice peptides sample of MW<3k was hydrolyzed by gastrointestinal enzymes to explore their antihypertensive activity change in vitro. The experiment conditions and results are shown in Table 6. The results demonstrated that antihypertensive activity of rice peptides slightly decreased after gastrointestinal enzyme hydrolysis, however, they still kept high ACE inhibitory activity even after hydrolysis for 4h. The result indicated that rice peptides may have antihypertensive effect when applied by oral administration to SHRs.

Table 6 ACE Inhibitory activity of rice peptides hydrolyzed by gastrointestinal enzymes in vitro

Experiment condition	ACE Inhibitory Rate(%)
Rice peptides	51.5
Rice peptides + pepsin, 2h @ 37°C, pH 1.8	46.8
Rice peptides + pepsin, 2h @ 37°C, pH 1.8, then + trypsin 2h @ 37°C, pH 8.0	38.8

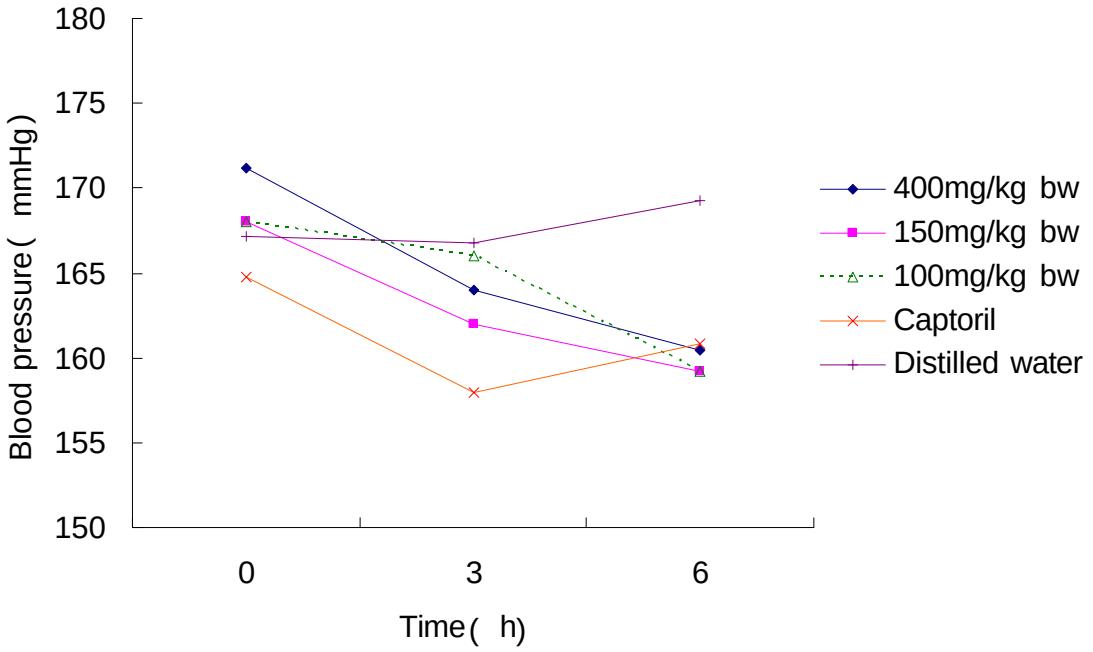

Fig. 13 Artery blood pressure of SHR by the administration of different rice peptides or Captopril

Fig. 13 show the artery blood pressure change of rats after oral feeding with rice polypeptides (for 100, 150, and 400 mg/kg body weight), respectively. It was found that the artery blood pressure of SHR significantly decreased after 3 h for different doses of oral feeding with rice polypeptides and Captopril. This result indicated that rice polypeptides have an antihypertensive effect in vivo.

CONCISE GENERAL SUMMARY OF CURRENT YEAR'S RESEARCH

This research investigated the potential of utilizing rice protein to develop of functional polypeptides and examine their antihypertensive effects. The following conclusions were made. Alcalase was the suitable proteases to digest rice protein for producing antihypertensive polypeptides product, and the optimal digestion conditions were thus: substrate content of 4% (m/v), Alcalase 7.5% (E/S), digestion temperature of 50^{o}C, and pH 9.5. Ultrasonic pretreatment of rice protein for antihypertensive polypeptides production led to a significant increase of inhibitory rate of hydrolysates to ACE up to 92.8% from 69.8% of control without ultrasonic pretreatment. Separation of polypeptides by using ultrafiltration with 10k, 5k, and 3k Dalton membranes in sequence indicated that the rice polypeptides with the MW of lower than 3k Dalton have the highest ACE-inhibitory activity. The next step of the research should develop pilot scale processing capability for producing a relatively large amount of product for more board functional feeling tests of animal and humans.

PUBLICATIONS OR REPORTS

N/A

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