

## PRODUCTION OF ANTIHYPERTENSIVE PEPTIDES BY ENZYMATIC ZEIN HYDROLYSATE FROM MAIZE-ZEA MAYS SSP. MEXICANA INTROGRESSION LINE

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### Abstract

Teosintes are essential gene reservoir for maize breeding improvement, among which *Zea mays* ssp. *mexicana* has many valuable traits deserved to be transferred into maize genetic background. In this study, one maize-teosinte introgression line SD00100 was selected from the population of *Zea mays* ssp. *mexicana* as wild parent. This introgression line manifested the outstanding agricultural traits similar to maize parent Ye 515 and alien genetic material was identified by genomic *in situ* hybridization (GISH). To produce bioactive peptides with potent angiotensin converting enzyme (ACE) inhibitory activity, zein extracted from endosperm meal was then undergone enzymatic hydrolysis with thermolysin and the hydrolysate was then filtered through a 3 kDa cut-off membrane. ACE inhibitory activity of permeate from Ye 515 and SD00100 was evaluated by RP-HPLC. The IC<sub>50</sub> values of the peptides obtained from maize parent and the introgression line were 96.9 µg/ml and 22.9 µg/ml, respectively, with significant difference between them. Our results showed that an outstanding inbred maize line was obtained for production of antihypertensive peptides as well as for further development of functional food.

**Keywords:** Maize, Introgression line, Zein, Peptide, Hypertension

### Introduction

Hypertension is a major risk factor for cardiovascular diseases including coronary heart disease, peripheral artery disease and stroke. Angiotensin-converting enzyme (ACE) is one main regulator of blood pressure converting angiotensin I to a potent vasoconstrictor, angiotensin II and hydrolyzing bradykinin, a vasodilator, into bradykinin (1-7) (Tom *et al.*, 2003). Thus, ACE inhibitors (ACEI), such as Captopril and Enalapril are extensively used to treat essential hypertension in clinic. However, the side effects of these drugs such as cough, exanthema, hyperkalemia and so on have troubled the patients seriously (Amir *et al.*, 2009; Vyssoulis *et al.*, 2001), and thus finding new type of ACE inhibitors from natural resources is a tremendous challenge for researchers.

Antihypertensive peptides with ACE inhibitory ability from food proteins have been proved to lower the blood pressure based on animal and clinical studies (Hernández-Ledesma *et al.*, 2011). To date, milk and animal proteins are main sources of antihypertensive peptides, while only few plants have been found containing this kind of bioactive peptide, such as chickpea (Yust *et al.*, 2003), rapeseed (Marczak *et al.*, 2003), soybean (Zhang *et al.*, 2006), peanut (Guang & Phillips 2009), buckwheat (Aoyagi, 2006) and wheat (Matsui *et al.*, 1999). Maize, one foundational food and forage worldwide, is planted in an area of 156 million hectares per year in nearly 100 countries. Its kernels supply many macro- and micronutrients necessary for human metabolic needs and provide about 15% of the world's protein and 20% of the world's calories (Nuss & Tanumihardjo, 2010). Among maize proteins, zein is the major type that provides about half of total kernel nitrogen; extensive attention has been paid to make full

use of maize protein, especially zein, to obtain antihypertensive peptides. ACE inhibitors from maize gluten (Suh *et al.*, 2003; Kim *et al.*, 2004; Huang *et al.*, 2011) and zein (Maruyama *et al.*, 1989; Miyoshi *et al.*, 1991a) by enzymatic hydrolysis have been reported in recent years. To get new plant materials with high output and generation efficiency of active peptide is the goal of this study.

*Zea mays* ssp. *mexicana*, a wild relative of cultivated maize, has valuable traits such as strong growth vigor, high protein content in the kernel and notable immune or resistance to multiple fungal diseases. We have developed a maize-*mexicana* population with outstanding lines (Wang *et al.*, 2008a; Wang *et al.*, 2012). The objective of the present work is to find new maize inbred line with high generation potential of ACE inhibitory peptides by introgressing exogenous genetic material; New inbred line will provide novel breeding materials for functional foods or nutraceuticals in the near future and thus expand the application of maize wild relatives for crop improvement.

### Materials and Methods

**Materials:** Maize elite inbred line Ye515, *Zea mays* ssp. *mexicana* (Fig. 1a) and introgression line SD00100 (Fig. 1b) selected from the maize-*mexicana* population (Wang *et al.*, 2008b) were kind gift from Professor Juren Zhang of Shandong University, China. SD00100 has some valuable traits such as big ears (Fig. 1c), long stay-green degree and high tolerance to biotic and abiotic stresses.

N-Hippuryl-His-Leu hydrate (HHL), ACE from rabbit lung, Hippuric acid (HA) and thermolysin were purchased from Sigma Chemical Co. DIG DNA Labeling kit for GISH analysis was from Roche Co. Other reagents are of analytical grade.

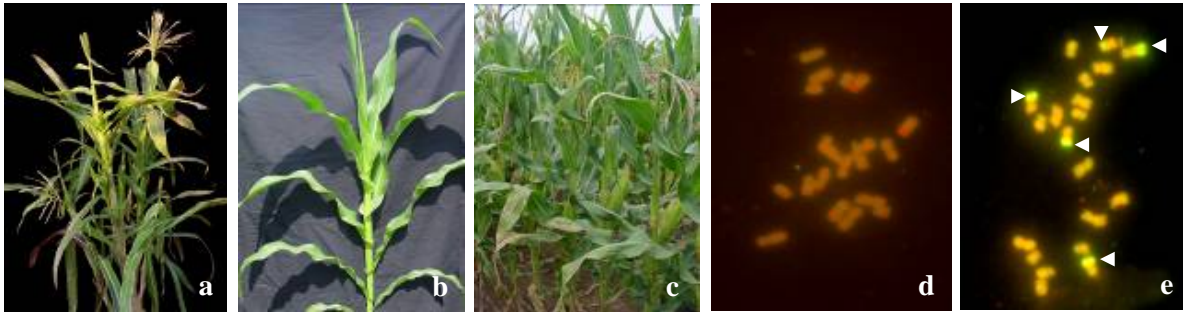


Fig. 1. Agricultural and cytogenetic traits of SD00100 and *Zea mays* ssp. *mexicana*. a, the plant of *Zea mays* ssp. *mexicana* with many tillers; b, the plant of line SD00100 showing similar characteristics to those of maize parent Ye 515; c, ear traits of line SD00100; d, Metaphase chromosomes of Ye515 after GISH analysis with genomic DNA from *Zea mays* ssp. *mexicana* as the probe and that of Ye 515 as blocking DNA. Red fluorescence all through the chromosomes with propidium-iodide counterstain manifested no hybridization signal was detected. e, GISH analysis of line SD00100 with Yellow-green hybridization signals carrying on the chromosomes (arrow) manifested introgression event had taken place in line SD00100.

**Measured agronomic traits:** All the lines were planted in Jinan (Shandong Province) at spring season for successive three generations with self-pollination to homogenize the genetic loci from 2008 to 2010. The following essential agricultural traits were recorded in 2010. Plant and ear heights were measured as the distance from the base of the plant to the top of the tassel and the node bearing the upper ear, respectively. Plant architecture was classified into compact, semi-compact and incompact type, respectively, according to Wang *et al.*, (2008a). Ear length (cm), ear circumference (cm) and kernel row number (integer) were also recorded after ears were harvested. The data were collected from five randomly sampled ears for each line.

**Identification alien genetic material by GISH:** To identify genetic material of *Zea mays* ssp. *mexicana* being integrated into SD00100, GISH was performed according to the method of Wang *et al.*, (2008a) with 80-fold blocking DNA. Briefly, qualified slides of root tips were denatured at 90°C for 2 min and the hybridization was performed overnight at 37°C. After post-hybridization wash, chromosomes were examined with an Olympus BX51 fluorescence microscope and the images were captured with Olympus 7070 digital camera.

**Amino acid analysis of endosperm flour:** The acid hydrolysis method was conducted to evaluate the amino acid contents in endosperm of Ye 515, *Zea mays* ssp. *mexicana* and line SD00100. Flour preparation of endosperm for all the samples followed the procedure of Wang *et al.*, (2008b). One hundred milligrams of sample was digested in 6 N HCl at 110°C for 24 h and then dried under vacuum. The samples were re-dissolved in 0.1 M lithium citrate buffer (pH 2.2). The subsequent amino acid was measured using amino acid analyzer Hitachi (L8900, Japan). Three samples were tested for each line.

**Preparation of zein and SDS-PAGE:** The flour was defatted twice with cold hexane and dried in vacuum for zein extraction. Zein was extracted by 70% ethanol containing 0.5% sodium acetate and 5%  $\beta$ -mercaptoethanol according to the procedure described by Paulis *et al.* (1977). The protein extracts were profiled by SDS-PAGE with 5% stacking gel and 15% separating gel and visualized by Coomassie Blue staining. The target zein,

dialyzed (3.5 KD molecular weight cut off) against distilled water for 24 h at 4°C, was vacuum freeze-dried for further analysis.

**Preparation of zein hydrolysate with thermolysin:** The lyophilized zein was digested enzymatically according to the method described by Kamath *et al.*, (2007) and Miyoshi *et al.*, (1991b). Samples (250 mg) were dissolved in 50 mM Tris-HCl (pH=8.0, 25 ml) containing 5mM CaCl<sub>2</sub> and then 25 mg thermolysin was added. The mixture was incubated with stirring at 37°C for 48 h. The reaction was stopped by heating in a boiling water bath for 5 min and the hydrolysate was centrifuged for 20 min at 4 °C and 3000 g. The supernatant was collected for further process.

**Ultrafiltration of enzymatic hydrolysates:** The hydrolysate solution was centrifugally filtered at 5000 rpm at 4°C for 2 h on Vivaspin centrifugal concentrators (Satorius, Germany) with the molecular weight cut-off of 3 kDa. The fractions with molecular weight  $\leq$  3 kDa were collected and freeze-dried for ACE inhibitory analysis.

**The assay of ACE inhibitory activity:** The peptides inhibitory activity of ACE was according to the method of Lin *et al.* (2011) with some modification. Briefly, A mixture (30  $\mu$ l) containing 0.1 M sodium borate buffer (pH=8.3) with 300 mM NaCl, 2 mU ACE and an appropriate amount of samples was preincubated for 10 min at 37°C. The reaction was initiated by adding 20  $\mu$ l HHL ( $2 \times 10^{-2}$  M), and terminated by adding 200  $\mu$ l acetonitrile. The release of hippuric acid (HA) from HHL was quantified by RP-HPLC on a C18 column (4.6 $\times$ 250 mm). Captopril and borate buffer solution were used as positive and blank control, respectively. The concentration of ACE inhibitory peptide required to inhibit 50% ACE activity under the above condition was defined as IC<sub>50</sub>, and at least five concentrations of each sample were used for the determination. Measurements were made in three duplications.

**Statistical analysis:** Data were manifested as mean  $\pm$  SD (standard error) and analyzed statistically by ANOVA using SPSS 10.0 program. If there was significant between-group effects, Waller and Duncan's multiple comparison test was applied to the means at the 0.05 level of significance.

**Table 1. Agricultural traits of Ye 515 and line SD00100<sup>a</sup>**

	Plant type	Plant height (cm)	Ear height (cm)	Kernel row number	Ear length	Ear circumference
Ye 515	Semi-compact	154.7±6.3b	46.2±4.8b	16.0±1.4a	13.5±1.1a	16.1±1.2a
SD00100	Semi-compact	211.8±15.0a	108.6±7.4a	15.6±1.7a	16.4±1.3a	16.9±1.4a

<sup>a</sup> Data collected from five plants of each analyzed line

Means with the different letters in each column were significantly different ( $p < 0.05$ ) by Duncan's multiple comparison test

**Table 2. Total amino acid content of Ye 515, *Zea mays* ssp. *mexicana* (ZM) and SD00100.**

Amino acid (mg / 100mg)	Ye 515	ZM	SD00100
Asp	0.73 ± 0.02b	1.43 ± 0.08a	0.59 ± 0.02c
Thr <sup>a</sup>	0.46 ± 0.02b	0.82 ± 0.04a	0.41 ± 0.02c
Ser	0.65 ± 0.05b	1.20 ± 0.06a	0.6 ± 0.040b
Glu	2.52 ± 0.07b	5.75 ± 0.30a	2.18 ± 0.09b
Gly	0.34 ± 0.00b	0.47 ± 0.02a	0.28 ± 0.01c
Ala	0.99 ± 0.02b	2.40 ± 0.13a	0.83 ± 0.06b
Cys	-	0.25 ± 0.02	-
Val <sup>a</sup>	1.21 ± 0.12a	1.37 ± 0.08a	1.24 ± 0.09a
Met <sup>a</sup>	0.28 ± 0.10b	0.46 ± 0.07a	0.23 ± 0.03b
Ile <sup>a</sup>	0.38 ± 0.03b	0.83 ± 0.04a	0.31 ± 0.05b
Leu*	1.88 ± 0.06b	4.98 ± 0.26a	1.63 ± 0.12b
Tyr	-	0.54 ± 0.10	-
Phe <sup>a</sup>	1.54 ± 0.11a	1.45 ± 0.09a	1.51 ± 0.10a
Lys	0.28 ± 0.04a	0.32 ± 0.03a	0.24 ± 0.03a
His <sup>a</sup>	0.38 ± 0.07b	0.61 ± 0.01a	0.38 ± 0.12b
Arg*	0.27 ± 0.03b	0.59 ± 0.04a	0.27 ± 0.07b
Pro	0.89 ± 0.02b	2.15 ± 0.25a	0.67 ± 0.04b
Total	12.79 ± 0.39	25.59 ± 1.44	11.36 ± 0.59

<sup>a</sup>Essential amino acid, - Not detected

Means with the different letters in each line were significantly different ( $p < 0.05$ ) by Duncan's multiple comparison test

## Results

**Agricultural traits of the lines:** Data on traits analyzed in both Ye 515 and SD00100 were listed in Table 1. Ye515 exhibited semi-compact plant type with the average plant height of 154.7 cm and ear position of 46.2 cm. *Zea mays* ssp. *mexicana* had many tillers with mixed plant architecture with long, shallow and lanceolate leaves. Its ear consisted of 2 interleaved rows of 6-12 kernels enclosed in a hard fruitcase. This wild relative of maize also manifested high resistance to insect pests and diseases except for maize smut disease. While line SD00100 showed semi-compact plant type which may inherited from cultivated maize parent Ye 515. The plant and ear height of SD00100 was significantly greater than those of Ye 515, which meant great bio-output from the introgression line. For ear-related traits, the kernel row number, ear length and ear circumference of SD00100 was 15.6, 16.4 and 16.9, respectively, VS 16.0, 13.5 and 16.1 for Ye 515. One outstanding ear character of Ye 515, an elite inbred line in Chinese agriculture, is large kernel row number, while there was no statistical difference between that of SD00100 and Ye 515 on this trait. These results showed that SD00100 has great yield potential and can be used for further food process.

**GISH analysis of line SD00100:** To identify the genetic structure of line SD00100, GISH analysis was conducted. Genomic DNA of *Zea mays* ssp. *mexicana* labeled with DIG-11-dUTP (Roche) was employed as DNA probe, and that of Ye 515 was used as blocking DNA. In the root tip cells of this introgression line, chromosome fragments of *Zea mays* ssp. *mexicana* were observed as yellow-greenish hybridization signals (Fig. 1e) while no signal was detected on all the chromosomes of Ye 515 (Fig. 1d). The result confirmed the alien chromatin had been integrated into maize background.

**Amino acid analysis of endosperm meal:** The endosperm hydrolyzed amino acid composition of both parents and SD00100 was shown in Table 2. All the 17 kinds of amino acid were detected in the wild parent, most of which were significantly higher than both Ye 515 and SD00100 except Val, Phe and Lys. While for Ye 515 and SD00100, 15 types of amino acids were detected except for Cys and Tyr. The essential amino acid content of SD00100 is not significantly smaller than that of Ye 515 besides Thr. The data indicated that the nutritional quality of SD00100 was not worse than Ye 515, while the biomass and kernel output for introgression line was obviously improved resulted from carrying alien genetic material.

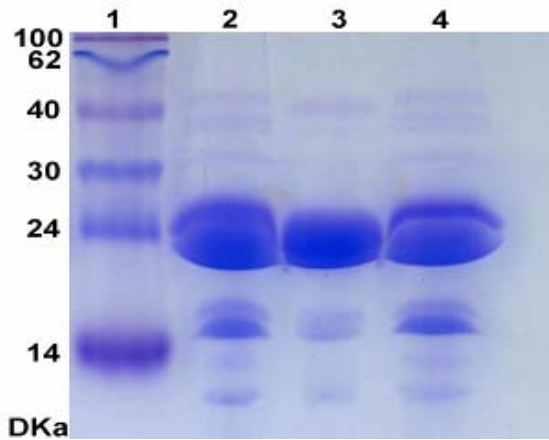


Fig. 2. SDS-PAGE analysis of zeins extracted from endosperm mill of both parent lines and SD00100. Lane 1 molecular weight standards; lane 2 Ye 515; Lane 3 *Zea mays ssp. mexicana*; Lane 4 line SD00100

**Extraction of zein from endosperm:** Proteins isolated by aqueous ethanol extraction containing 0.5% sodium acetate and 5%  $\beta$ -mercaptoethanol from endosperm mills of all the three lines contained a mixture of zeins. Fig. 2 showed the electrophoretic profiles of both parents and offspring introgression line. All the four types of zeins- $\alpha$ -zein (19 and 22 kD),  $\beta$ -zein (14 kD),  $\gamma$ -zein (16 and 27

kD) and  $\delta$ -zein (10 kD) were detected suggesting the method of extraction of zeins was effective. However, there were some different electrophoretic patterns among them. *Zea mays ssp. mexicana* manifested less or no 14, 27 kD and higher molecular mass bands (> 30 kD). The electrophoretic pattern of SD00100 was similar to that of maize cultivated parent as for bands and intensity.

**Analysis of peptides ACE inhibitory activity:** HPLC method was used to determine the ACE inhibitory reactions. The retention times of the substrate HHL and the product HA were respectively 5.7 (Fig. 3a) and 10.1 min (Fig. 3b). Complete baseline separation of HA and HHL was achieved in 5 min. The produced content of HA was reduced by 66.8 % because of the inhibition of ACE activity by captopril with the content of  $5 \times 10^{-8}$  M (Fig. 3c), which was in accordance with the reference report. Zein hydrolysate of Ye 515 and SD00100 also showed some extent inhibitory activity (Fig. 3d). The  $IC_{50}$  values of bioactive peptides from cultivated maize and introgression line were  $96.9 \pm 6.7$   $\mu$ g/ml and  $22.9 \pm 12.9$   $\mu$ g/ml (Fig. 4.), respectively, which were significantly different at  $P = 0.05$  level. This meant that enzymatic hydrolysis of zein is an effective way to release peptides able to exert anti-hypertension activity, and introgression line SD00100 carrying alien genes could be a better material to generate bioactive peptides.

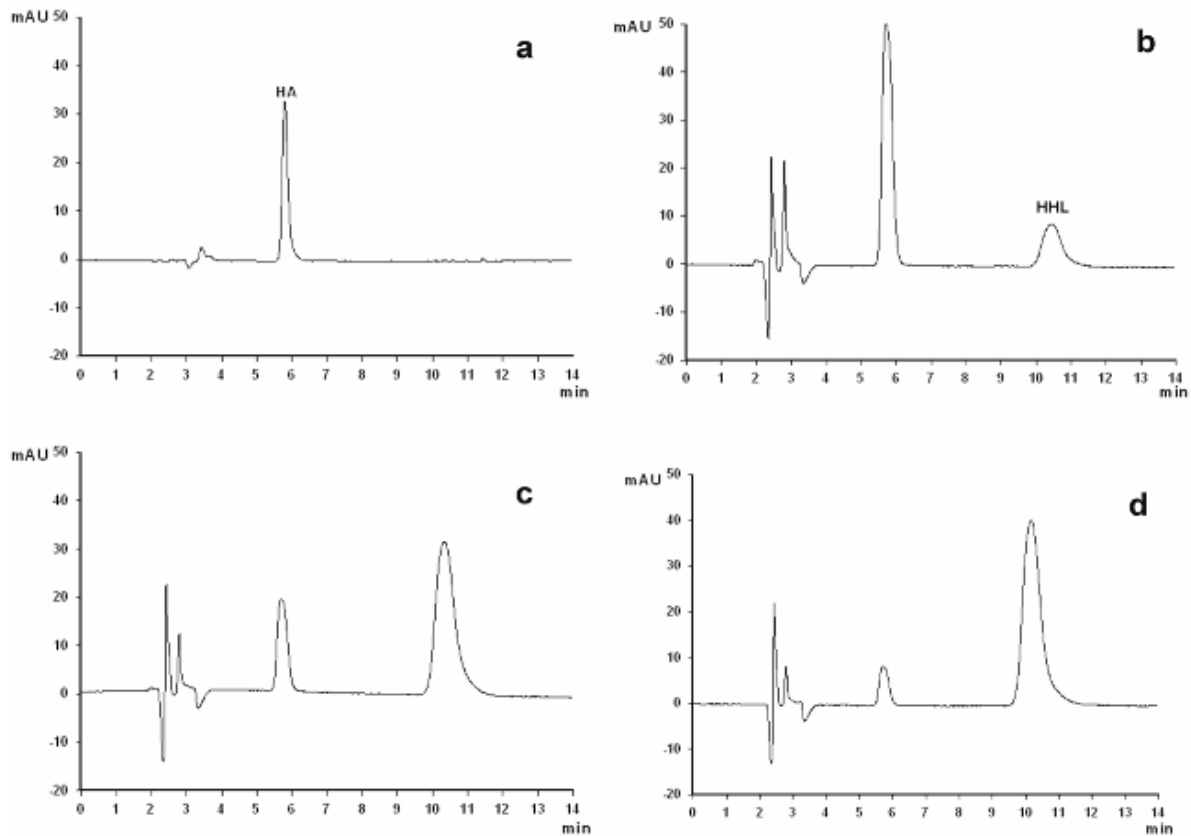


Fig. 3. HPLC profile of the detected samples a: HA, b: negative control (brotate buffer), c: captopril, d: zein hydrolysate of SD00100 at content of 289.2  $\mu$ g/ml. Retention times of the substrate HHL and the product formed by ACE hydrolysis HA were 10.1 and 5.7 min, respectively.

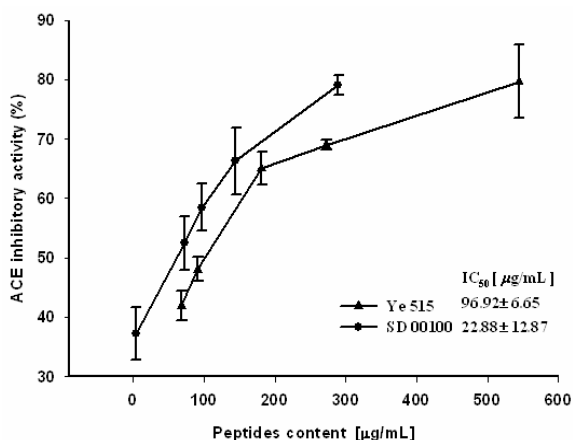


Fig. 4. The IC<sub>50</sub> of Ye 515 and SD00100 zein hydrolysates ( $\leq 3$  kDa) for ACE and significant difference was detected at  $p = 0.05$  level.

## Discussion

ACE inhibitory substances are widely used to lower the blood pressure of hypertensive patients. Food or natural resources derived ACE inhibitors have safety advantages over synthetic compounds, however efforts to develop novel more effective peptides are still required, as these peptides have lower antihypertensive activity than captopril, enalapril and so on (Kim *et al.*, 2010). Bioactive peptides can be enzymatically released from food protein during protein processing and/or gastrointestinal digestion. Many hypertensive peptides from various food protein have been reported (Hernández-Ledesma *et al.*, 2011).

There are limited reports on using maize to generate bioactive peptide with ACE inhibitory activity. Huang *et al.*, (2011) had produced peptides with ACE-inhibitory activity from corn gluten meal, the by-product of starch industry, which was rich in zein and glutelin by using Alcalase and the IC<sub>50</sub> of peptides (molecular weight smaller than 3 kDa) was 0.29 mg protein/ml. Anti-hypertensive peptides from gluten using 6 different commercial proteases were also obtained (Suh *et al.*, 2003). As we all know, zein is the major storage protein of corn and comprises about 45-50% of the total protein in corn. Its application includes use in fiber, adhesive, coating, ceramic, textile and so on for the ability of tough, glossy, hydrophobic grease-proof and the resistance to microbial attack. It is not used directly for human consumption due to its negative nitrogen balance and poor solubility in water (Shukal *et al.*, 2001). Making functional food with diverse physiological function from zein may be desirable to find new utilization of it. Through this research, inbred with high potent against ACE activity was obtained by introgression hybridization. The rather high ACE inhibitory ability for both Ye 515 and SD00100 may attribute to the high key amino acids content concerning for the target ACE activity, such as Phe, Pro, Leu. Enzymatic  $\alpha$ -zein hydrolysate had been obtained with rather high *in vitro* and *in vivo* antihypertensive activity (Miyoshi *et al.*, 1991; Miyoshi *et al.*, 1991b). Thus, the next work we should do is to further fraction peptides in the hydrolysate by ion-exchange chromatography and/or high-performance liquid chromatography and identify their

composition and sequence by mass spectrometry and detect new functional bioactive peptides with potential application for treating hypertension.

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