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Design and production of genetically modified soybean protein with anti-hypertensive activity by incorporating potent analogue of ovokinin(2–7)

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Abstract The potent anti-hypertensive peptide, RPLKPW, has been designed based on the structure of ovokinin(2–7). The sequence encoding this peptide was introduced into three homologous sites in the gene for soybean β-conglycinin α′ subunit. The native α′ subunit as well as the modified, RPLKPW-containing α′ subunit were expressed in *Escherichia coli*, recovered from the soluble fraction and then purified by ion-exchange chromatography. The RPLKPW peptide was released from recombinant RPLKPW-containing α′ subunit after in vitro digestion by trypsin and chymotrypsin. Moreover, the undigested RPLKPW-containing α′ subunit given orally at a dose of 10 mg/kg exerted an anti-hypertensive effect in spontaneously hypertensive rats, unlike the native α′ subunit. These results provide evidence for the first time that a physiologically active peptide introduced into a food protein by site-directed mutagenesis could practically function in vivo even at a low dose.

Key words: Ovokinin; Anti-hypertensive peptide; Soybean β-conglycinin; Genetically modified food; Spontaneously hypertensive rat

1. Introduction

Hypertension is one of the most common lifestyle-related diseases. These diseases being closely associated with diet, it is conceivable that food is conducive to preventing and ameliorating such diseases as an adjunct to drug therapy. Therefore, we tried to develop a novel food protein with anti-hypertensive activity for the dietetics of hypertension.

Previously, we isolated an anti-hypertensive hexapeptide, ovokinin(2–7) (RADHPF), from a chymotryptic digest of ovalbumin [1]. Ovokinin(2–7) induces nitric oxide meditated vasorelaxation. At an oral dose of 10 mg/kg, ovokinin(2–7) significantly lowered the systolic blood pressure in spontaneously hypertensive rats (SHRs) but failed in normotensive Wistar–Kyoto rats (WKYs). These results indicate that ovokinin(2–7) possesses an intriguing property to have little effect on normal blood pressure. However, at least 600 mg/kg of ovalbumin is required to exert its anti-hypertensive activity when given orally to SHRs assuming that the peptide is cleaved from the protein thoroughly.

To obviate such an inconvenience, our efforts were centered on potentiating the anti-hypertensive activity of ovokinin(2–7) by replacing the amino acid residues in the peptide [2]. Among various ovokinin(2–7) analogues so far designed, RPLKPW showed the highest activity, thereby significantly lowering the systolic blood pressure of SHRs after oral administration at a dose of 0.1 mg/kg, a comparable dose to those of synthetic anti-hypertensive drugs (Yamada et al., Peptides, submitted). Moreover, the blood pressure in WKYs was not affected by RPLKPW even at a dose of 1 mg/kg, indicating that this peptide possesses an ideal, potent anti-hypertensive activity.

In order to utilize the potent anti-hypertensive peptide or RPLKPW as a food for the dietetics of hypertension, attempts were made to incorporate the peptide into food protein by genetic engineering. RPLKPW contains four different residues from the original parental peptide ovokinin(2–7). This required our extensive search for other candidate food proteins except for ovalbumin as the target. Three RPLKPW-like sequences were found in the extension region of soybean β-conglycinin α′ subunit (Fig. 1). β-Conglycinin, one of the major soybean storage proteins [3,4], is composed of three subunits, α, α′ and β (543, 559, 416 residues, respectively) [5]. These homologous sequences were referred to as RPQHPE, RPRQPH and RPHQPH, corresponding to residues 16–21, 46–51 and 86–91 of the β-conglycinin α′ subunit, respectively. In this report, these three RPLKPW-like sequences in β-conglycinin α′ subunit were changed to RPLKPW by site-directed mutagenesis of the cDNA encoding the α′ subunit. The modified protein was produced using an *Escherichia coli* expression system to evaluate the anti-hypertensive activity in SHR.

2. Materials and methods

2.1. Bacterial strain, medium and plasmid

E. coli HMS174(DE3) (Novagen) was used as the host. Luria–Bertani (LB) medium (pH 7.0) consisted of 1% bactotryptone, 0.5% yeast extract and 1% NaCl. The expression plasmid used here was pECα′

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Abbreviations: APMSF, (*p*-amidinophenyl)-methylsulfonyl fluoride; GM, genetically modified; HPLC, high performance liquid chromatography; LB, Luria–Bertani; ODS, octadecyl silica; PAGE, polyacrylamide gel electrophoresis; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto rat; TFA, trifluoroacetic acid

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[5], in which the cDNA encoding the β-conglycinin α′ subunit was subcloned into the expression vector pET21d(+) (Novagen) between the *Nco*I and *Bam*HI sites.

2.2. Animals

Male SHRs (16–22 weeks old) were purchased from Shimizu Laboratory Supplies, Kyoto, Japan. Rats were fed with SP chow (Funabashi Farm) and water ad libitum, and housed in a temperature-controlled room with a 12-h light/dark cycle. This study was performed in accordance with the guidelines for the care and use of laboratory animals of Kyoto University.

2.3. Site-directed mutagenesis

The expression plasmid pECα′ was used as the template for site-directed mutagenesis. To change three RPLKPW-like sequences in β-conglycinin α′ subunit, or RPQHPE, RPRQPH and RPHQPH, to RPLKPW, and change the amino acid residues next to the N-terminus of each of these three sequences to arginine or tryptophan, two stages of site-directed mutagenesis with synthetic oligonucleotide primers were performed on the respective regions in pECα′ encoding RPLKPW-like sequences using a QuikChange™ Site-Directed Mutagenesis kit (Stratagene). The primers used were as follows: (the base sequences for the mutation are underlined) for RPQHPE, 5′-CCA CGA CCA CGA CCA TTG AAG CCG TGG AGG GAA CGT CAG CAA CAC-3′ and 5′-GTG TTG CTG ACG TTC CCT CCA CGG CTT CAA TGG TCG TGG TCG TGG-3′ (first stage) and 5′-GAA GGT CAA ATT CCA CGA CGA CGA CCA TTG AAG CCG-3′ and 5′-CGG GTG GAA TGG TCG TCG TCG TGG AAT TTG ACC TTC-3′ (second stage), for RPRQPH, 5′-CCA TTC CCA CGC CCA CTC AAA CCT TGG CAA GAG GAA GAG CAC GAG-3′ and 5′-CTC GTG CTC TTC CTC TTG CCA AGG TTT GAG TGG GCG TGG GAA TGG-3′ (first stage) and 5′-CGT CCA TTC CCA TTC TGG CGC CCA CTC AAA CCT TGG C-3′ and 5′-G CCA AGG TTT GAG TGG GCG CCA GAA TGG GAA TGG ACG-3′ (second stage) and for RPHQPH, 5′-GAA CAC CCA CGC CCA CTC AAA CCT TGG CAA AAG GAA GAG GAA AAG-3′ and 5′-CTT TTC CTC TTC CTT TTG CCA AGG TTT GAG TGG GCG TGG GTG TTC-3′ (first stage) and 5′-GAA CGT GAA CAC CGA CGC CCA CTC AAA CCT TGG CAA AAG GAA GAG GAA AAG C-3′ and 5′-G CTT TTC CTC TTC CTT TTG CCA AGG TTT GAG TGG GCG TCG GTG TTC ACG TTC-3′ (second stage). The mutated pECα′ was transformed into *E. coli* XL1-Blue (Stratagene), and the transformants were cultured in 3 ml of LB medium containing 50 μg/ml ampicillin at 37°C overnight. After harvesting the cells by centrifugation, the amplified plasmids were extracted. The mutated DNA sequences were confirmed by the dideoxy chain-termination method of Sanger et al. [6], using a DNA sequencer (Applied Biosystems, Model 310) and an ABI dye terminator cycle sequencing kit with *AmpliTaq* polymerase FS (Perkin Elmer, Applied Biosystems).

2.4. Expression and detection of recombinant native and RPLKPW-containing α′ subunits in E. coli

Flasks of 700 ml of LB medium containing 50 μg/ml ampicillin were inoculated with 7 ml of full-grown cultures of HMS174(DE3) harboring the expression plasmid pECα′ or mutated pECα′ encoding the RPLKPW-containing α′ subunit and cultured at 37°C using an orbital incubator at 200 r.p.m. (Sanyo, Model MIR-220R). At A_{600} = 0.6–1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce expression of the native and RPLKPW-containing α′ subunits. Following induction, culture was continued at 150 r.p.m. for about 42 h at 20°C. The induced cells were harvested by centrifugation at 6000×*g* for 10 min and stored at −80°C. Aliquots of the cells were boiled in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol and 0.2 M 2-mercaptoethanol), and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 11% (m/v) acrylamide [7].

2.5. Purification of recombinant native and RPLKPW-containing α′ subunits from E. coli

All procedures were carried out at 4°C.

2.5.1. Extraction of the expressed protein from E. coli cells. The frozen cells were resuspended in buffer A (35 mM sodium phosphate, pH 7.6, 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)-methylsulfonyl fluoride (APMSF), 3 μg/ml leupeptin and 0.02% NaN_3) in a volume of 7 ml per gram of cells (wet weight), and disrupted by sonication on ice. Insoluble materials were separated from the soluble fraction by centrifugation at 20 000×*g* for 30 min.

2.5.2. Fractionation with ammonium sulfate. The soluble crude extract was adjusted to 40% (for the native α′ subunit) or 20% (for the RPLKPW-containing α′ subunit) saturation with solid ammonium sulfate and stirred for more than 30 min. After centrifugation at 20 000×*g* for 30 min, the supernatant was adjusted to 65% (for the native α′ subunit) or 50% (for the RPLKPW-containing α′ subunit) saturation and stirred overnight. The precipitated protein was collected by centrifugation at 20 000×*g* for 30 min, and resuspended in buffer B (35 mM sodium phosphate, pH 7.6, 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM APMSF, 3 μg/ml leupeptin and 0.02% NaN_3) in a small volume (up to 10 ml). The suspension was dialyzed overnight against 3×1 l of buffer C (35 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM APMSF, 3 μg/ml leupeptin and 0.02% NaN_3).

2.5.3. Q Sepharose column chromatography. The dialysate was clarified by centrifugation at 20 000×*g* for 30 min, and the resulting protein solution (up to 450 mg protein) was fractionated by anion-exchange high performance liquid chromatography (HPLC) on a Hi-Load 26/10 Q Sepharose high performance column (Amersham Pharmacia Biotech) equilibrated with buffer C. The column was initially washed with buffer C until the A_{280} of the eluate dropped to a stable baseline, and the elution was performed with a linear gradient of 0.1–0.6 M NaCl over a period of 250 min. The flow rate was 4 ml/min and the fraction size was 6 ml. Fractions containing the recombinant native and RPLKPW-containing α′ subunits were identified by SDS–PAGE and pooled. The pooled material was adjusted to 80% saturation with solid ammonium sulfate and stirred overnight. After centrifugation at 20 000×*g* for 30 min, the precipitated native and modified α′ subunits were dissolved in distilled water containing 0.1 M NaCl, pH 7.5, in a volume of ∼10 ml and dialyzed against 3×2 l of the same solution for 48 h and an additional 2×3 l of distilled water overnight. The resulting suspension was freeze-dried and stored at −20°C. The purified native and RPLKPW-containing α′ subunits thus obtained were used for further analysis.

2.6. Protein measurement

Proteins in the samples were determined according to the method of Bradford [8] using bovine serum albumin as a standard.

2.7. Amino acid sequence analysis

The N-terminal sequence of recombinant RPLKPW-containing α′ subunit was determined using a Procise 492 Protein Sequencer (Applied Biosystems), whereas the C-terminal sequence was determined using a Procise 494-C Protein Sequencer (Applied Biosystems).

2.8. In vitro digestion of recombinant RPLKPW-containing α′ subunit

Recombinant RPLKPW-containing α′ subunit (1 mg/ml) was adjusted to pH 8.0 and digested with trypsin and chymotrypsin (E/S = 1/400 (w/w)) for 3 h at 37°C. The reaction was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 0.1%, and then the reaction mixture was centrifuged.

2.9. Purification and detection of RPLKPW from the digestion of recombinant RPLKPW-containing α′ subunit

The digest was fractionated by reversed-phase HPLC on an octadecyl silica (ODS) column (Cosmosil 5C18-AR-II, 4.6×150 mm, Nacalai Tesque). The column was eluted with a linear gradient of acetonitrile (1%/min), containing 0.1% TFA at a flow rate of 1 ml/min. Elution was monitored at 280 nm. The expected fraction containing RPLKPW was collected according to the elution position of the synthetic peptide. The fraction was concentrated in a centrifugal concentrator, and applied to a liquid chromatograph mass spectrometry (Mariner, PerSeptive Biosystems) or a 492 protein sequencer (Applied Biosystems).

2.10. Measurement of the blood pressure after oral administration of recombinant native and RPLKPW-containing α′ subunits to SHRs

The recombinant native and RPLKPW-containing α′ subunits were emulsified with 30% egg yolk in saline and administered orally to SHRs using a metal zonde in a volume of 0.5 ml [9]. The systolic

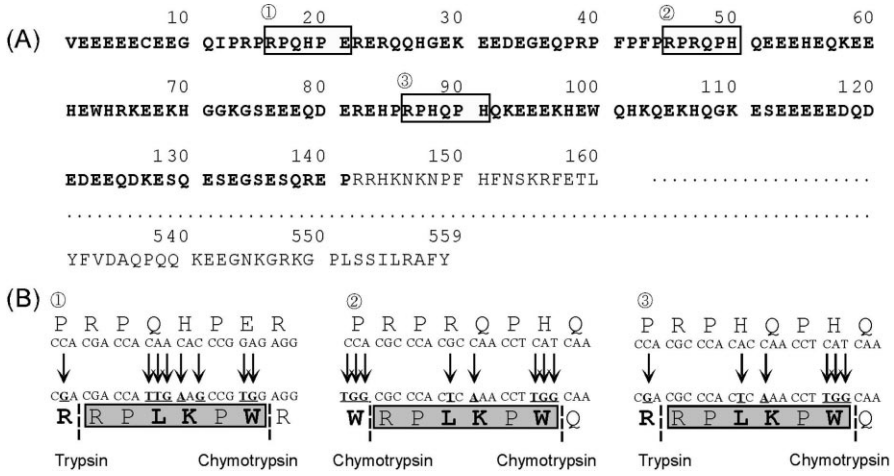

Fig. 1. Three RPLKPW-like sequences in a soybean β-conglycinin α′ subunit (A) and the design of site-directed mutagenesis (B). Bold letters in (A) correspond to the extension region. Putative trypsin or chymotrypsin cleavage sites are indicated by the dotted line in (B).

blood pressure was measured by the indirect tail cuff method using an MK-2000 (Muromachi Kikai). Following oral administration, the blood pressure was measured every 2 h.

2.11. Data analyses

Results of blood pressure measurements are expressed as means ± S.E.M. Statistical evaluation was performed by analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Introduction of RPLKPW sequences into β-conglycinin α′ subunit

Three RPLKPW-like sequences including RPQHPE, RPRQPH and RPHQPH, corresponding to residues 16–21, 46–51 and 86–91 of β-conglycinin α′ subunit, respectively, were changed to RPLKPW by site-directed mutagenesis of the cDNA encoding the α′ subunit in the expression plasmid pECα. All these sequences are preceded by Pro residues. To facilitate the release of RPLKPW from the modified α′ subunit in the intestine after ingestion of the protein, the Pro residues should also be changed to arginine or tryptophan (Fig. 1). This modification allowed the RPLKPW sequences to be released by trypsin and chymotrypsin. As a result, four residues were replaced in each site.

3.2. Expression and detection of recombinant native and RPLKPW-containing α′ subunits in E. coli

Expression of recombinant native and RPLKPW-containing α′ subunits in *E. coli* cells harboring individual expression plasmids was assessed by SDS–PAGE. Densitometric scanning of the gels indicated that both the native and RPLKPW-containing α′ subunits accumulated in *E. coli* at a level of around 15% of the total bacterial proteins (Fig. 2, lanes 2 and 6). The mobility of the RPLKPW-containing α′ subunit on SDS–PAGE was slightly faster than that of the native molecule. Immunoblotting analysis revealed that both

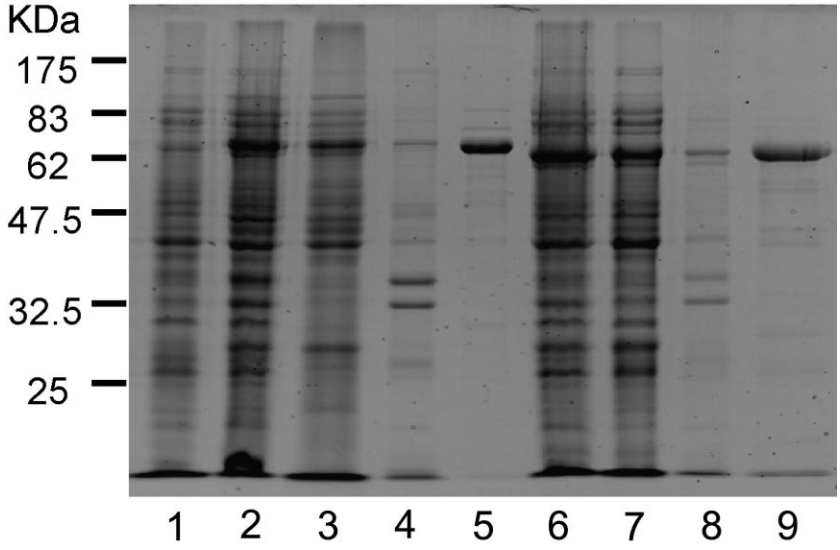

Fig. 2. SDS–PAGE analysis of the native and the RPLKPW-containing β-conglycinin α′ subunit. Recombinant native (lanes 2–5) and modified (lanes 6–9) were expressed in *E. coli* HMS174(DE3). Lane 1, non-transformed *E. coli* proteins; lanes 2 and 6, total cell proteins after expression; lanes 3 and 7, soluble cell proteins; lanes 4 and 8, insoluble cell proteins obtained by sonicating the cells; lanes 5 and 9, purified native and modified α′ subunits by Q Sepharose column.

the native and the RPLKPW-containing α′ subunit were recognized by anti-recombinant α′ subunit antiserum (data not shown). Both the native and RPLKPW-containing α′ subunits were recovered mostly in the soluble fractions (Fig. 2, lanes 3 and 7).

Recombinant native and RPLKPW-containing α′ subunits in the soluble fractions were purified using ammonium sulfate as the first step. The native α′ subunit was precipitated in the 40–65% ammonium sulfate fraction, whereas the RPLKPW-containing α′ was precipitated in the 20–50% fraction. After dialysis, the pure α′ subunits were obtained by anion-exchange chromatography using a Q Sepharose column (Fig. 2, lanes 5 and 9, Fig. 3). As a result, 13.9 mg of the native α′ subunit and 14.6 mg of the modified protein were each obtained from 2.8 l of culture.

To verify that the obtained RPLKPW-containing α′ subunit still remained in the complete form, the N- and C-terminal amino acid sequences of the protein were analyzed. Both the sequences determined here (seven residues of the N-terminus and three residues of the C-terminus) coincided with those of the native α′ subunit reported previously [5]. About three quarters of both the native and the modified protein had an additional N-terminal methionine residue.

3.3. *In vitro digestion of recombinant RPLKPW-containing α′ subunit and detection of RPLKPW*

To determine whether RPLKPW could be released from recombinant RPLKPW-containing α′ subunit as expected, the modified α′ was digested with trypsin and chymotrypsin in vitro. After fractionation of the digest by HPLC using an ODS column, the fraction with the same retention time as that of synthetic RPLKPW was analyzed by liquid chromatograph mass spectrometry, and a peak with the same molecular mass as RPLKPW was detected. For further verification, the fraction was applied to a protein sequencer. The sequence determined here coincided completely with RPLKPW. The overall yield of the finally purified RPLKPW was ∼1%.

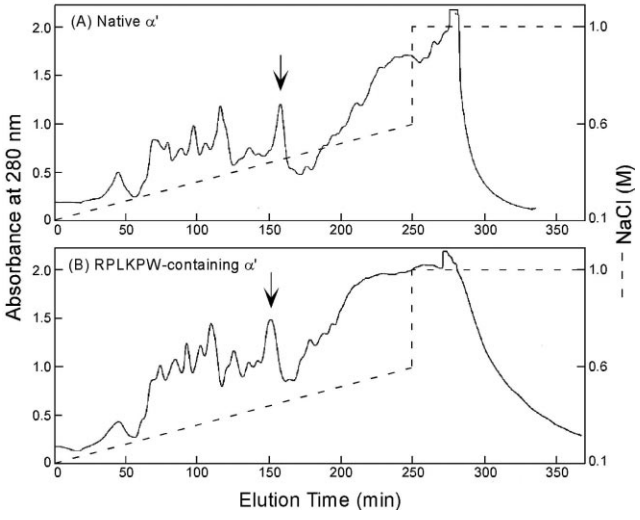

Fig. 3. Purification of recombinant α′ subunits from the total *E. coli* proteins by anion-exchange HPLC on a Q Sepharose column. (A) The native α′ subunit. (B) The RPLKPW-containing α′ subunit. Fractions containing the α′ subunits are indicated by arrows.

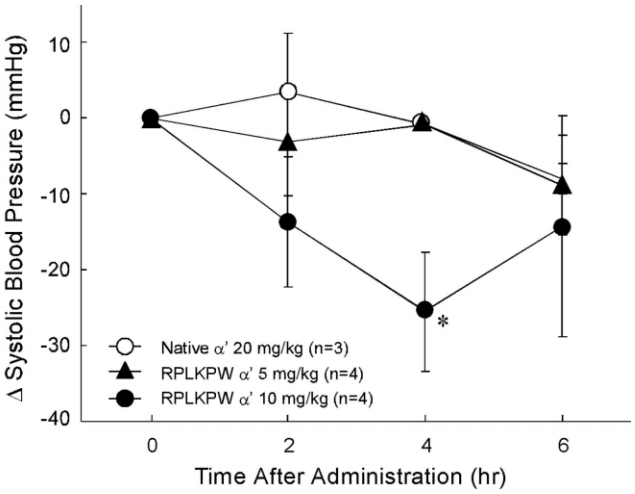

Fig. 4. Anti-hypertensive activity of RPLKPW-containing β-conglycinin α′ subunit after oral administration in SHRs. The numbers of experiments are shown in parentheses. Changes in systolic blood pressure from zero time are expressed as means ± S.E.M. Statistical evaluation was performed by ANOVA. *Significantly different from the native α′ group (*$P < 0.05$).

3.4. *Anti-hypertensive activity of recombinant RPLKPW-containing α′ subunit*

The results of in vitro digestion of recombinant RPLKPW-containing α′ subunit suggested that RPLKPW could be released by intestinal trypsin and chymotrypsin after ingestion of the non-digested protein, thereby allowing prompt investigation of the anti-hypertensive effects after its oral administration to SHR.

As shown in Fig. 4, orally administered recombinant RPLKPW-containing α′ subunit at a dose of 10 mg/kg significantly lowered the systolic blood pressure of SHRs 4 h after administration. The lowered blood pressure returned to the basal level 6 h post-administration. At a dose of 5 mg/kg, the RPLKPW-containing α′ subunit failed to show the anti-hypertensive effect, indicating that the minimal effective dose of the RPLKPW-containing α′ subunit is 5–10 mg/kg. In contrast, however, the native α′ subunit at a dose of 20 mg/kg had no effect on the blood pressure.

4. Discussion

In this study, three sets of RPLKPW, an orally active, potent anti-hypertensive peptide that has been designed on structure–activity relationship study of ovokinin(2–7), were introduced into the homologous sequences of soybean β-conglycinin α′ subunit by site-directed mutagenesis. After oral administration to SHRs, recombinant RPLKPW-containing α′ subunit successfully lowered the blood pressure at a dose of 10 mg/kg. As the minimal effective dose of the RPLKPW was 0.1 mg/kg when given orally to SHR, at least 30% of the integrated RPLKPW in the RPLKPW-containing α′ subunit should be released in the gastrointestinal tract of SHRs orally medicated with this protein. On the other hand, ovalbumin exerted its anti-hypertensive activity at a dose of as high as 2 g/kg (data not shown), indicating that the RPLKPW-containing α′ subunit designed here has a 200-fold potent anti-hypertensive effect compared to ovalbumin. Coupled with

these facts, the RPLKPW-containing α′ subunit proved to have a potent anti-hypertensive effect as a designed food protein.

The maximum decrease in the blood pressure was noted 4 h after administration of the recombinant RPLKPW-containing α′ subunit. The time-course pattern of the onset was consistent with those after administration of the free RPLKPW peptide, indicating that the peptide was liberated from the protein rapidly after ingestion. All the three RPLKPW-containing sequences might be vulnerable to easy cleavage due to their specific location on the extension region of the α′ subunit, which is hydrophilic by itself and projected on the surface of the molecule [5].

Although the molecular mass of recombinant RPLKPW-containing α′ subunit was not significantly different from that of the native protein, the RPLKPW-containing α′ subunit showed a slightly faster electrophoretic mobility relative to the native protein on SDS–PAGE. However, analysis of both N- and C-terminal amino acid sequences suggested that no processing had occurred on the RPLKPW-containing α′ subunit. The mobility shift could be explained by some conformational change of the RPLKPW-containing α′ subunit. The RPLKPW-containing α′ subunit was, however, recovered in the soluble fraction in *E. coli*, suggesting that the protein was folded normally. Moreover, the extension region of the α′ subunit was suggested to have a minor role in proper folding [5]. This indicates that no significant changes occurred in the overall conformation of the RPLKPW-containing α′ subunit, although further investigations such as circular dichroism measurement are required.

It is noteworthy that the intact RPLKPW-containing α′ subunit exhibited anti-hypertensive effects, thereby being indicative of the RPLKPW-containing α′ subunit as an anti-hypertensive food protein. It remains to be investigated whether recombinant RPLKPW-containing α′ subunit could be produced and accumulated properly in plant tissue. Glycinin, another major soybean storage protein, was shown to have the correct conformation after expressed in *E. coli* [10] and accumulate stably in rice [11], potato [12] and tobacco seed [13] even after modification in its variable region. Consequently, we regard rice especially as another candidate to produce the RPLKPW-containing α′ subunit besides soybean. Most genetically modified (GM) crops marketed to date have been developed to bring great profit to farmers by introduction of genes conferring properties such as insect resistance and herbicide tolerance. As the natural consequence, with the expectation that a second generation of GM crops bringing about benefit to the consumers would be attained [14], various crops with beneficial health effects are now being explored [15–18]. Although further investigations with regard to the safety evaluation (e.g. allergenicity, chronic toxicity etc.) remain to be performed, incorporation of the RPLKPW-containing α′ subunit into crops could assume a greater role in establishing novel GM crops for dietetics of hypertension in the near future.

This study showed for the first time that physiologically active peptides introduced in a food protein by site-directed mutagenesis could practically function in vivo. These observations will open new avenues of study on the physiologically active peptides derived from food proteins, leading to the second generation of GM crops with beneficial health effects.

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