Depressor Effect of Wheat Germ Hydrolysate and Its Novel Angiotensin I-Converting Enzyme Inhibitory Peptide, Ile-Val-Tyr, and the Metabolism in Rat and Human Plasma

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We report here the antihypertensive effect of wheat germ (WG) hydrolysate and its dominant peptide, Ile-Val-Tyr (IVY), with potent angiotensin I-converting enzyme (ACE) inhibitory activity. The toxicity test of AG50W fraction purified from the WG hydrolysate and IVY in ddY mice revealed that 1 week median lethal concentrations of AG fraction and IVY were less than 100 and 10 mg/kg, respectively. As a result of an intravenous administration test of both inhibitors in spontaneously hypertensive rat (SHR), the mean arterial blood pressure (MAP) significantly decreased with the dose; the MAP reduction of 10.3 and 19.2 mmHg was observed at a dose of 50 mg/kg of AG fraction and 5 mg/kg of IVY, respectively. In addition to this behavior, the MAP gradually decreased after the 5 mg/kg of IVY injection (time to give a maximum reduction; 8 min), and the reduction was held for 20 min. By using rat and human plasma, IVY was found to be metabolized by the action of aminopeptidase in plasma to form a subsequent ACE inhibitor, Val-Tyr (VY). Thus, the intake of IVY as a physiologically functional food would serve in the lowering of blood pressure (BP) by the combined depressor effect of itself and its metabolite after the absorption.

Key words angiotensin I-converting enzyme inhibition; hypertension; wheat germ; metabolism

Wheat germ (WG), which is rich in vitamin E, is a good source of protein, though it has low usability in food industry. In our previous report,1 we found that the hydrolysis of WG protein by Bacillus licheniformis alkaline protease is a sufficient treatment to exert a physiological functional property. The typical ability was a powerful retardation of the action of angiotensin I-converting enzyme (ACE, EC3.4.15.1). Namely, 0.5 wt% 8 h alkaline protease hydrolysis after 3.0 wt% 3 h a-amylase treatment of defatted WG showed a potent ACE inhibitory activity, with an IC50 value of 0.37 mg-protein/ml. The activity of WG hydrolysate was markedly increased by octadecyl silica (ODS) and by subsequent AG50W cation-exchange purifications (denoted as AG fraction, IC50: 0.018 mg-protein/ml).

Many investigators have already pointed out the efficacy of the in vitro depressor action of natural ACE inhibitors or hydrolysates.2–6) Recently, Hata et al.6 reported that the oral administration of 100 ml of fermented milk containing 1.5 mg Val-Pro-Pro (IC50: 9 μm) and 1.1 mg of Ile-Pro-Pro (IC50, 5 μm) in hypertensive subjects for 4 weeks showed a significant reduction of blood pressure (BP), by 9.4 mmHg of systolic BP (SBP) and 6.9 mmHg of diastolic BP (DBP). As we have already reported,7 sardine muscle hydrolysate also showed a potent depressor effect in mild hypertensive subjects, in which the BP was significantly lowered for 4-week run experiment (ΔSBP/ΔDBP; 9.7 mmHg/5.3 mmHg). In this hydrolysate, the peptide, Val-Tyr (VY), with the IC50 value of 5.2 μm, was identified to contribute ACE inhibition.8,9) The results that some natural ACE inhibitory peptides had in vivo antihypertensive effects strongly led us to investigate whether the WG hydrolysate exerts such effects in vivo as reported for in vitro experiments.1)

From the WG hydrolysate, we have isolated sixteen ACE inhibitory peptides with IC50 values of less than 20 μm.1) Among these peptides, we have identified Ile-Val-Tyr (IVY) as a main contributor to the in vitro ACE inhibition of the hydrolysate, because of the powerful ACE inhibitory activity (IC50 of 0.48 μm, high content of 0.26 wt% in it, and high resistance to digestion.1) In addition to this finding, IVY possesses a VY sequence with a depressor action in hypertensive human.7) Thus, in this paper, the in vivo depressor effect of IVY, as well as AG fraction, on spontaneously hypertensive rat (SHR) was also investigated. We have tried to speculate the in vivo inhibitory action of IVY through the metabolic process in rat and human blood.

MATERIALS AND METHODS

Materials and Reagents WG was supplied from Taiyo Seifun Co. (Fukuoka, Japan). Enzymes used in this study were Bacillus α-amylase from Amano Seiyaku, and Bacillus licheniformis alkaline protease from Novo (2.4L, type FG). Purified rabbit lung ACE was purchased from Sigma. ODS-AQ 120-S50 and AG50W-X8 resins were from YMC and Bio-Rad, respectively. Cosmosil 5C18-AII and Cosmosil 5P columns were from Naracalai Tesque. Bestatin as a synthetic aminopeptidase inhibitor was obtained from Sigma. All other reagents used in this study were purchased from Naracalai Tesque.

Assay for ACE Inhibitory Activity and Kinetic Studies ACE inhibitory activity was determined by the proposed 2,4,6-trinitrobenzene sulfonate (TNBS) method.10) For each assay, 25 μl of ACE inhibitor and 50 μl of hippuryl-l-histidyl-l-leucine (Hip-His-Leu) as a substrate in a borate buffer (pH 8.3) containing 200 mm NaCl were incubated with 50 μl of ACE (12.5 mU/ml) at 37°C for 1 h. The reaction was stopped by adding 125 μl of 0.5 M HCl, the solution was adjusted to pH 9.12, and 25 μl of 0.1 m TNBS in a 0.1 m
Na$_3$HPO$_4$ solution was then added. After incubation at 37°C for 20 min, 4.5 ml of 4 mM Na$_2$SO$_4$ in a 0.2 m NaH$_2$PO$_4$ solution was added, and the absorbance of the yielded trinitrophenyl (TNP)-His-Leu complex at 416 nm was measured with a Shimadzu UV-1200 spectrophotometer. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the assayed conditions was defined as the IC$_{50}$ value.

The kinetic study was performed under the same conditions described above except for the incubation time of 30 min. The concentration range of Hip-His-Leu was 0.37—5.8 mm. The Ki of the inhibitor for ACE was determined from the x-intercept of the secondary replots of the Lineweaver-Burk plots in the presence of the inhibitor.

Preparation of WG Hydrolysate The alkaline protease hydrolysis of WG and subsequent AG50W purification were performed according to our previous report. Briefly, defatted WG by n-hexane at 80°C for 5 h was subjected to a 3.0 wt% 3 h α-amylase treatment, followed by 0.5 wt% 8 h enzymatic hydrolysis. The hydrolysate treated with activated charcoal powder was purified with a YMC ODS-AQ 120-S50 column (3.5×13 cm). A 10% ethanol eluate was then put on an AG50W-X8 cation-exchange (H$^+$) disposable column, and eluted with 1.0 M NH$_4$OH solution. The eluted AG fraction was used for further investigation in this study.

Toxicity Test in Mouse The toxicity of IVY and AG fraction by single intravenous administration was tested in male ddy mouse from Kiwa Laboratory Animals Institute, Co. (32—36 g, 6-week-old). Twenty ddy mice were used and divided into two groups. The dosages of IVY and AG fraction were 10 and 100 mg/kg, respectively. Ten ml/kg of the physiological saline solution was injected into the tail vein. Body weight, motility, and the lethal tolerance against both ACE inhibitors were observed for 1 week after the administration.

Measurement of Antihypertensive Effect in Rat SHRs, 12 weeks old, were supplied by Biochemical and Pharmacological Laboratories, Inc. (Tondabayashi, Japan). Twenty SHRs (310—350 g) with tail SBP of >180 mmHg were used, and were divided into four groups. The SHR was anesthetized with pentobarbital sodium (40 mg/kg), and the carotid artery was cannulated for recording the mean arterial blood pressure (MAP) with a Nihon Koden AP-641G blood pressure transducer. IVY (2 and 5 mg/kg), and AG fraction (25 and 50 mg/kg) dissolved in physiological saline were subjected to the intravenous administration test; 1 ml/kg of the solution was injected into the tail vein, and the MAP was recorded for 30 min after the injection. The MAP in SHR is expressed as mean±S.D. (%) (n=5 rats), and the statistical analysis was performed by Student’s paired t-test to evaluate the difference in MAP before and after administration of IVY or AG fraction.

Degradation Test on IVY in Rat and Human Plasma The SHR or normotensive human plasma was prepared by centrifuging blood samples at 1500×g for 15 min at 4°C, which were then drawn into chilled vacutainer tubes containing sodium heparin. To 250 µl of ACE inhibitor solution, 20 µl of plasma was added, and the solution was incubated at 37°C for several intervals up to 24 h. The ACE inhibitor solution was prepared by adding 0.8 mM IVY to a 0.1 M Tris-maleate buffer (pH 7.4) containing 1 mM CaCl$_2$. The incubation was stopped by adding 12.5 µl of 0.1 m EDTA-2Na solution. The amount of IVY or the product was determined with the reversed-phase HPLC (Shimadzu LC-9A instrument, Kyoto, Japan; column: Cosmosil 5C18-ARII, 4.6×250 mm) in the linear gradient mode of acetonitrile (CH$_3$CN) (5—9%, 30 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.4 ml/min (220 nm). A newly observed peak was identified by a Shimadzu PPSQ-21 protein sequencer. Prior to the determination, the calibration curve of IVY and its product was measured using their standards under the same HPLC conditions described above. Each result is expressed as the mean (nmol/assay volume)±S.E. (%) of three independent experiments.

RESULTS

Toxicity Test in Mouse The toxicity of the AG fraction of the WG hydrolysate and its prominent ACE inhibitory peptide, IVY, were tested in mouse at dosages of 100 mg/kg of AG fraction and 10 mg/kg of IVY, respectively. Although data are not shown, there was no remarkable change in the body weight of ten ddy mice between the before group (AG group; 33.4±1.3 g, IVY group; 33.4±1.4 g) and the group 1 week after single intravenous administration (AG group; 36.8±1.6 g, IVY group; 35.8±2.5 g). No unusual motility of any of the mice after injection was observed. Furthermore, no mouse was dead during the 1 week-study, indicating that the 1 week median lethal concentration (1 wk-LC$_{50}$) was more than 10 and 100 mg/kg in IVY and AG fractions, respectively. Thus, the administration of <10 mg/kg of IVY or <100 mg/kg of AG fraction would provide no toxicity in animals.

Antihypertensive Effect of AG Fraction and IVY in SHR From the above mentioned 1 wk-LC$_{50}$ value, intravenous administration tests of AG fraction and IVY in SHR were done at dosages of 25 and 50 mg/kg, and 2 and 5 mg/kg, respectively. Table 1 summarizes the changes in MAP before and after the injection of AG fraction and IVY. As a result, the MAP in SHR decreased after the injection of the AG fraction, and a significant (p<0.01) MAP reduction of 10.3 mmHg was observed at a dose of 50 mg/kg. As for IVY, a significant (p<0.05) reduction of MAP (19.2 mmHg) was also observed at a dose of 5 mg/kg. For both inhibitors, the higher dosage resulted in much more MAP reduction in SHR: the reduction ratio of AG fraction at 25 mg/kg, 4.6%; 50 mg/kg, 5.4%; IVY at 2 mg/kg, 3.1%; 5 mg/kg, 10.4%. Figure 1 shows the time course of MAP reduction ratio of AG fraction (50 mg/kg dose) and IVY (5 mg/kg) during the 30 min-protocol. Although the time required for a maximum lowering of MAP (t$_{max}$) varied with inhibitor (50 mg dose of AG fraction, 13 min; 5 mg dose of IVY, 8 min), the MAP reduction for both inhibitors was held for at least 10 min after the corresponding t$_{max}$; namely, an immediate return to the normal state was not observed with either inhibitor.

Degradation in Rat and Human Plasma To speculate the ACE inhibitory effect of IVY after absorption, in vitro metabolic experiments were done using rat plasma. Figure 2 shows the change in concentration of IVY in rat plasma within 24 h of incubation. The production behavior of its fragments was also represented in the same Figure. The fragments from IVY were identified by the protein sequencer. As
Table 1. Changes in BP of SHR before and after Intravenous Administration of AG Fraction and IVY

<table>
<thead>
<tr>
<th>Dose</th>
<th>BP (mmHg) Before</th>
<th>BP (mmHg) After</th>
<th>Reduction ratio (%)</th>
<th>Time after injection (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG fraction 25 mg/kg</td>
<td>186.8±4.04</td>
<td>178.3±8.89</td>
<td>4.6±5.6</td>
<td>5</td>
</tr>
<tr>
<td>AG fraction 50 mg/kg</td>
<td>189.6±2.06</td>
<td>179.3±6.41*</td>
<td>5.4±3.5</td>
<td>13</td>
</tr>
<tr>
<td>IVY 2 mg/kg</td>
<td>184.0±11.0</td>
<td>178.3±16.3</td>
<td>3.1±4.5</td>
<td>13</td>
</tr>
<tr>
<td>IVY 5 mg/kg</td>
<td>184.7±8.49</td>
<td>165.5±14.5**</td>
<td>10.4±6.2</td>
<td>8</td>
</tr>
</tbody>
</table>

*a) AG fraction by AG50W-X8 cation-exchange chromatography was prepared from the 10% ethanol ODS eluted fraction of the alkaline protease WG hydrolysate. Details are described in the Materials and Methods section. BP is shown as mean±S.D., n=5. Significant differences from before administration by paired Student t-test: *p<0.05, **p<0.01.

![Graph showing BP reduction over time](image)

Fig. 1. Reduction Behavior of BP after Intravenous Administration of AG Fraction and IVY in SHR

AG fraction (---), 50 mg/kg dose; IVY (- - -), 5 mg/kg dose.

![Graph showing concentration of metabolites and IVY](image)

Fig. 2. Degradation of IVY with ACE Inhibitory Activity and Production of Its Fragments in Rat Plasma within 24 h of Incubation

The incubation of IVY (250 ml of 0.8% milk peptide in a 0.1 M Tris-maleate buffer (pH 7.4) containing 1 mM CaCl₂) was initiated with 20 µl of plasma. Open bars represent the total amount of remaining IVY and fragments produced over each incubation interval. Each bar represents the mean±S.E. (%) of three independent experiments.

![Graph showing concentration of metabolites and IVY](image)

Fig. 3. Degradation of IVY with ACE Inhibitory Activity and Production of Its Fragments in Normotensive Human Plasma within 24 h of Incubation

The incubation conditions are the same as in Fig. 1. Each bar represents the mean±S.E. (%) of three independent experiments.

As can be seen, IVY began to degrade immediately after the incubation with plasma, and half of the degradation (t₁/₂) was observed at ca. 4 h of incubation. After incubation for 24 h, IVY completely disappeared within our experimental conditions. As for the production behavior, only two fragments of VY and Tyr were detected by HPLC analysis. VY was mainly formed within the first 15 min of incubation, then accumulated in plasma with the incubation of IVY thereafter. Interestingly, 24.4±3.1 nmol/assay volume of VY with about 1/9 initial concentration of IVY (196.2±4.8 nmol/assay volume) still remained in plasma after incubation for 24 h. The first detection of Tyr was observed after incubation for 1 h, and increased with time in rat plasma. The same study was done in normotensive human plasma. As shown in Fig. 3, similar metabolic behavior to that in rat plasma was obtained in human plasma. However, a greater accumulation of VY (95.3±0.9 nmol/assay volume) was observed than in rat plasma at 24 h incubation. Also, only 1.5 h incubation (t₁/₂) was needed for degrading half the initial concentration of IVY (199.3±5.6 nmol/assay volume).

In order to clarify the metabolism of IVY in both types of plasma, the effect of the addition of bestatin as an aminopeptidase inhibitor(13) on its degradation was examined under the same conditions described above (Fig. 4). As a result, the degradation of IVY in human plasma was significantly (p<0.01) suppressed in the presence of 1.0 µM bestatin: the concentration of IVY after incubation for 2 h without bestatin was 72.4±5.9 nmol/assay volume, and with bestatin was 197.4±6.0 nmol/assay volume. No significant difference in the amount of IVY before or after the incubation with bestatin strongly suggested that the metabolism of IVY in plasma predominantly proceeds from a sequential cleavage of N-terminal amino acids through the action of aminopeptidases. The same suppression effect by bestatin was obtained in rat plasma, though data were not shown.

Table 2 summarizes the Kᵢ value and degradation rate of IVY and its predominant product, VY, in rat and human plasma. As a result, VY from IVY still had 1/30 ACE inhibition power (Kᵢ; 3.03 µM), compared to its precursor (IVY, Kᵢ;
no report with respect to the toxicity or antigenicity of smaller natural peptides or oligopeptides. With saridine hydrolysate with an average peptide chain length of 2.67, we have had negative results in the active systematic anaphylaxis (ASA) test, as well as in the passive cutaneous anaphylaxis (PCA) test in guinea pigs by oral administration.\textsuperscript{19} In contrast, Hartlbe and Leuschen\textsuperscript{19} revealed the high toxicity of a mixture of low molecular weight peptides from porcine spleen, in which the IC\textsubscript{50} was 0.112 mg/kg in rats for a single dose. However, it is noteworthy that di- and tri-peptides have nutritional implications as a nitrogen source.\textsuperscript{17,18} Therefore, less than 100 mg/kg dose of AG fraction (<10 mg/kg dose of IVY) would be applicable as a physiologically functional food material.

As a result of the intravenous administration tests of AG fraction and IVY in SHR, both inhibitors were found to lower the MAP and the reduction of MAP increased with dose (Table 1). In particular, the injection of 5 mg/kg dose of IVY resulted in a maximum MAP reduction ratio of 10.4%. The reduction ratio of IVY was almost consistent with that of the 50 mg/kg dose of YY (9.9%), as reported elsewhere.\textsuperscript{19} By considering that YY had a 10-fold higher IC\textsubscript{50} value of 5.2 \textmu M\textsuperscript{1} than that of IVY (IC\textsubscript{50} 0.48 \textmu M\textsuperscript{1}), IVY appears to have stronger in vivo depressor ability than YY by a factor of 10 or more. Based on the facts of the in vivo depressor effect of YY in human\textsuperscript{19} and SHR\textsuperscript{20} by oral administration, IVY would exert a sufficient antihypertensive effect with very small dosage amounts in human.

According to the MAP reduction behavior of IVY (Fig. 1), the MAP gradually decreased after the IVY injection (t\textsubscript{max} 8 min), and the reduction was held for 20 min. Contrary to this finding, we have already reported that YY had an acute depressor effect in SHR; then, an immediate return (about 5 min after injection) to the normal state was observed.\textsuperscript{19} This longer depressor effect of IVY than YY could be explained by the metabolism of IVY in plasma. Based on the fact that half of the degradation of Gly-Gly-Gly absorbed in human blood was observed after ca. 1 h of postdose,\textsuperscript{21} the degradation of IVY (t\textsubscript{1/2} ca. 4 h in rat plasma, Fig. 2) seems to be slower. In addition, IVY subsequently degraded to YY with an antihypertensive effect (Figs. 2 and 3). This indicated that the longer depressor effect of IVY would be caused by the combined depressor effect of IVY and its prominent metabolite, YY. Then, the apparent total ACE inhibition induced by IVY and its subsequent active fragment, YY, in human blood was estimated by the amounts of remaining IVY and YY produced, as shown in Fig. 2. As a result of extrapolation (Fig. 5), the long term ACE inhibitory effect of IVY over 24 h of postdose in human would be expected by the successive production of YY.

The study of inhibition by bestatin (Fig. 4) revealed that the enzyme involved in the metabolism of IVY in rat and human plasma was aminopeptidases. Furthermore, the sum of remaining IVY and fragments produced over each incubation interval corresponded to the initial amount of IVY in both plasmas (Figs. 2 and 3). Therefore, IVY in rat and human plasma would degrade via the same metabolic pathway represented in Fig. 4. A large difference in the remaining amounts of YY at the 24 h of incubation and in the t\textsubscript{1/2} of IVY between both plasma (Figs. 2 and 3) would be due to the difference in the aminopeptidase activity involved in the IV

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Table 2. Kinetics of IVY and Its Product, YY, in Human and Rat Plasma

<table>
<thead>
<tr>
<th></th>
<th>Degradation rate (K_i^{\text{a}}(\mu M))</th>
<th>Rat plasma</th>
<th>Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVY</td>
<td>0.10</td>
<td>18.0</td>
<td>46.7</td>
</tr>
<tr>
<td>YY</td>
<td>3.03</td>
<td>14.2</td>
<td>16.4</td>
</tr>
</tbody>
</table>

\(a\) \(K_i\) value of ACE inhibitor was determined at 37°C for 30 min-incubation. The concentration range of His-His-Leu was 0.35—5.8 mm. \(b\) Twenty \mu l of plasma sample was added to 250 \mu l of 0.8 mm inhibitor solution (0.1 M Tris-maleate buffer (pH 7.4) containing 1 mm CaCl\textsubscript{2}). The degradation ratio was determined for 15 min-incubation. The amount of IVY or YY was assayed by reversed phase HPLC, column, column: C18-AQRI, 4.6×250 mm; eluent, linear gradient mode of CH\textsubscript{3}OH (5—95%, 30 min) in 0.1% TFA at a flow rate of 0.4 ml·min\textsuperscript{-1} (220 nm).

0.10 \mu M. On the other hand, IVY was more liable to degradation in human plasma (46.7 nmol/min/ml-plasma) rather than in rat plasma (18.0 nmol/min/ml-plasma). Little difference in the YY degradation rate between both plasmas was observed.

**DISCUSSION**

In our previous report,\textsuperscript{1} we have revealed that the AG fraction of alkaline protease WG hydrolysate had the ability to retard the action of ACE (IC\textsubscript{50} 0.018 mg-protein/ml). The activity was comparative to the activity (IC\textsubscript{50} 0.015 mg protein/ml) of sardine muscle hydrolysate, with a depressor effect in mildly hypertensive subjects.\textsuperscript{7,14} Thus, to elucidate the in vivo antihypertensive effect of the WG hydrolysate, an intravenous administration test of its AG fraction was done in SHR. IVY (2 and 5 mg/kg doses) was also tested as a main ACE inhibitory contributor.\textsuperscript{1} The dosage of AG fraction (25 and 50 mg/kg) was in line with the human-protocol study of the 2 g intake of the sardine hydrolysate (ca. 33 mg/kg in human body weight).\textsuperscript{7} Prior to this protocol, their toxicity at a dosage of 100 mg/kg of AG fraction (10 mg/kg of IVY) was examined in ddy mouse. As a result, no remarkable changes in body weight or unusual motility were observed during the 1 wk-protocol. As far as we know, there has been
bond cleavage. According to the report by Allard et al., aminopeptidase M present in blood would be probable, in which YI or VY was metabolized to amino acids by the enzyme in cultured mouse spinal cord cells.

In conclusion, the present study demonstrated that IVY as a main ACE inhibitor in the WG hydrolysate lowered the MAP in SHR. The effect would be caused by the combined depressor effects of itself and its dominant metabolite, VY. Based on these findings, the antihypertensive effect of WG hydrolysate and IVY on human subjects is now under investigation.

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