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Inhibitory Efficacy and Biological Variability of Tryptophan Containing Dipeptides on Human Plasma Angiotensin Converting Enzyme Activity

Sherif Khedr, Melanie Martin and Andreas Deussen*

Institute of Physiology, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, 01307 Dresden, Germany

Abstract

Background: Hypertension remains a major unsolved health problem with life threatening consequences. A potential innovative prophylactic approach against its development may be supplementation of daily food with natural antihypertensive compounds. In our work we tested the efficacy of novel tryptophan containing dipeptides from whey protein with respect to their inhibitory potency on the angiotensin converting enzyme (ACE) as well as their stability in human plasma.

Methods: ACE activity from rabbit lung and human plasma as well as recombinant human ACE activity were measured using benzoyl-glycyl-histidyl-leucine as a substrate. Product formation of hippuric acid and histidyl-leucine was assessed by UV-HPLC. The stability of the peptides was quantified in human plasma and, in case of incubation with recombinant human angiotensin converting enzyme, in buffer. Variability of ACE inhibiting activity of peptides was assessed via analysis of coefficient of variation with particular reference to true inter-individual variability corrected for methodological and analytical errors.

Results: Isoleucine-tryptophan (IW) had a stronger ACE inhibiting potency (IC_{50} of 1.9 and 38.8 µmol/l with rabbit lung and human plasma ACE, respectively) than other tested peptides, i.e. glutamic acid-tryptophan (EW), tryptophan-leucine (WL) and tryptophan-glutamic acid (WE). However, stability of IW showed a high degree of variability in plasma of different volunteers. In contrast EW, WL, WE were relatively stable in plasma and no major inter-individual variability was observed. Inhibition of plasma proteases extended plasma half-life of IW significantly. ACE did not contribute to peptide elimination.

Conclusion: Milk whey protein represents a valuable source for bioactive peptides with ACE inhibitory potency that can be used as a food supplement to prevent, or in combination with other pharmaceutical agents to treat hypertension. However, in addition to the ACE inhibitory efficacy the stability of these peptides must be considered for *in vivo* ACE inhibition.

Keywords: Hypertension; Angiotensin converting enzyme; Bioactive peptides; Whey protein; Isoleucine-tryptophan

Abbreviations

ACE: Angiotensin converting enzyme; AT1: Angiotensin type 1-receptor; CV: Coefficient of variability; EW: Glutamic acid-tryptophan; IC_{50}: Half maximal inhibitory concentration; IW: Isoleucine-tryptophan; PI: Proteases inhibitor; RAS: Renin–angiotensin system; rhACE: Recombinant human ACE; W: Tryptophan; WE: Tryptophan-glutamic acid; WL: Tryptophan-leucine

Introduction

Hypertension is a major unsolved cardiovascular problem and one of the leading causes of morbidity and mortality in developing as well as developed countries [1]. Activation of the renin–angiotensin system (RAS) is a leading mechanism in the genesis of this disease [2]. Inhibition of this hormonal axis either by ACE inhibitors or angiotensin type 1-receptor (AT1) blockers ameliorates activation of AT1 receptors on smooth muscles reducing vasoconstriction and, by acting on cells in the zona glomerulosa of the adrenal cortex, reduces aldosterone secretion and thereby salt and water retention. As a consequence, such pharmaceutical agents powerfully lower arterial blood pressure and induce vessel wall remodeling [3].

A potential approach for hypertension prevention could be supplementation of daily food products with effective natural compounds which interact with the RAS. Several natural peptides hydrolyzed from complex food proteins have been shown to exert ACE inhibitory effects and as a consequence they may lower arterial blood pressure [4]. Milk protein is one of the richest sources for many bioactive peptides with different biological actions. Antihypertensive peptides attracted attention in the past and, thus, have been extensively studied [5].

When considering such bioactive peptides for potential usage as food additives, besides their effect on ACE activity, their stability in human plasma must be considered. Dipeptides are typically hydrolyzed by action of peptidases and tryptophan containing peptides are prone in particular to hydrolysis by aminopeptidase W and aminopeptidase N [6]. In the current communication we tested in a comprehensive set of experiments the ACE inhibitory potency of tryptophan containing dipeptides glutamic acid-tryptophan (EW), isoleucine-tryptophan (IW), tryptophan-leucine (WL) and tryptophan-glutamic acid (WE), assessed whether these dipeptides exhibit differences in their plasma stability and tested whether individual blood donors differ in their hydrolytic activity for tryptophan containing dipeptides.

***Corresponding author:** Prof. Andreas Deussen, Department of Physiology , Medical Faculty , Dresden University of Technology, D-01307 Dresden, Germany, Tel: +49-351-458-6030; Fax: +49-351-458-6301; E-mail: Andreas.Deussen@tu-dresden.de

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Materials and Methods

Materials

Chemicals used included L isoleucyl-L tryptophan, L tryptophan-L leucine, L glutamic acid-L tryptophan, L tryptophan-L glutamic acid and hippuryl-histidyl-leucine (all: purity>95%, Bachem, Bubendorf, Switzerland), captopril (purity>98%, Sigma Aldrich, Munich, Germany), methanol HiPerSolv CHROMANORM (LC-MS grade, VWR, EC), ortho-phosphoric acid 85% (Merck KGaA, Darmstadt, Germany), rabbit lung ACE (Sigma Aldrich, Munich, Germany), recombinant human ACE (R&D Systems Inc., Minneapolis, MN), and protease inhibitor cocktail (Roche, Mannheim, Germany).

Plasma collection and storage

The procedure was approved by the institutional ethics board (Permission EK 299082014). Blood samples (20 ml) for analysis of ACE activity and peptide stability were taken by venipuncture. Blood samples were collected in S Monovette® tubes containing 9 N trisodium citrate (Sarstedt, Nuembrecht, Germany), centrifuged (4°C, 1500 rpm, 20 min), then stored at -20°C until ACE activity determination.

ACE activity

ACE activity was assessed by incubating 30 µL of rabbit lung ACE (supplied as lyophilized powder reconstituted in PBS to give an activity of 0.02 U/ml) or 60 µL of human plasma with 100 µL of ACE specific substrate hippuryl-histidyl-leucine (5 mmol/l). Additionally 10 µL of PBS (blank) or peptides dissolved in PBS were added and incubated for 1 hour at 37°C. The enzymatic reaction was stopped by adding 190 µL HCl (0.33 mol/l).

Recombinant Human ACE (10 µg) supplied as lyophilized powder was dissolved in 3 ml PBS. Enzyme activity was assessed by incubating 20 µl of the dissolved enzyme with 50 µl hippuryl-histidyl-leucine (5 mmol/l) for 1 hour at 37°C. The reaction was stopped by adding 100 µL HCl (0.33 mol/l). Samples were stored at -20°C until analysis of ACE activity.

Peptide stability

Stability of peptides in plasma was tested by incubating 10 µL of the respective peptide solution (concentration 100 µmol/l) with 60 µL of plasma diluted in 100 µL of PBS for 1 hour, then the reaction was stopped using 190 µL HCl (0.33 mol/l). Samples were stored at -20°C until analysis.

Reversed phase high performance liquid chromatography (RP-HPLC)

The amount of peptides remaining and hippuric acid formed were measured via RP-HPLC. For determination of compounds, 20 µl samples were injected into the HPLC system (Waters Alliance 2695, Eschborn, Germany) equipped with an UV-detector (Waters 2487, λ = 228 nm) using a Waters XBridge RP18 column (3.5 µm, 150 x 4,6 mm). Starting conditions were ultrapure water containing 0.15% H_3PO_4 (solvent A) and methanol augmented with 0.15% H_3PO_4 (solvent B) at a flow rate of 1 ml/min. Elution was achieved by a gradient of solvent B as follows: 3 to 56% in 9 min, 56 to 97% in the next 4 min, 97 to 3% in the following 5 min. Re-equilibration was held for 3 min at 3% solvent B. Total run time was 21 min. Because of co-elution of the peptide EW with hippuric acid, in this case the gradient of solvent B was set to 3% for 4 min, 3 to 35% in 23 min, 35 to 97% in the next 5 min, staying at 97% for 3 min, 97% to 3% in the following 2 min. Re-equilibration was held for 2 min at 3% solvent B (solvents and flow rate were maintained). Total run time was 39 min.

Calculations and Statistics

Coefficients of variability were calculated from the percentage ratio of standard deviation (SD) over the mean value, according to King et al. [7], the observed variability was assumed to be the sum of the contributing independent variances as outlined in results. Methodological variability was defined as the error in sample measurement caused by HPLC, the lowest hierarchical error. It was calculated by analyzing the same sample three times. The coefficient of variability was then calculated by dividing the SD of these measurements by the mean value.

Handling variability was obtained from plasma ACE activity measurements of a single volunteer performed on three different plasma samples. Each sample was prepared separately and analyzed by HPLC. The error of this procedure had two sources: first, the error of the sample handling and second, the error of the HPLC analysis. The true error of the handling procedure was deduced as follows:

$$CV_{handling} = (CV^2_{total\ error} - CV^2_{method})^{1/2} (1)$$

Observed (whole) variability was obtained using samples from several individuals. Each sample was prepared and subjected to enzyme activity measurements, then analyzed using HPLC. Here, the observed variability had four sources of error: inter-individual differences, temporal differences, and differences resulting from sample preparation and HPLC analysis as stated:

$$CV^2_{observed} = CV^2_{inter\text{-}individual} + CV^2_{temporal} + CV^2_{handling} + CV^2_{method} (2)$$

Sigma plot 12 was used to calculate the IC_{50} (i.e. the concentration of the compound that induces 50% inhibition in the enzyme activity). Data is reported as mean value ± SD. To test for group differences ANOVA and Tukey's test for correction of multiple comparisons were used. A p value<0.05 was taken to indicate a significant difference.

Results

The inhibitory potency for tryptophan containing dipeptides EW, IW, WL, and WE was investigated on rabbit lung ACE activity. A concentration-dependent reduction of ACE activity was observed for all tested peptides, except WE which showed minimal inhibitory effect (Figure 1). IW had the highest ACE inhibitory potency (IC_{50} = 1.9 µM) among the tested peptides. It was, however, less effective in comparison to captopril which was used as positive control (IC_{50} = 3.8 nM) (Table 1).

Further investigations were done with human plasma ACE from different volunteers. Again, EW, IW and WL showed significant ACE inhibiting potency, while WE was not effective. IW showed the highest ACE inhibiting potency (IC_{50} = 38.8 µM). Unexpectedly, however, IW and captopril showed inhibitory effects which varied markedly among different volunteers. This was reflected by considerable standard deviations at all concentrations. In contrast, the other peptides tested showed little variability of ACE inhibition in plasma of different volunteers (Figure 2).

The variability was expressed as coefficient of variation ($CV_{observed}$). Because ACE activity in human individuals is reported to be stable [8] and this agreed well with own results for ACE activity testing in plasma samples collected on three successive days from the same volunteer (data not shown), temporal variability was assumed to be negligible and equation (2) may be reduced to:

$$CV^2_{observed} = CV^2_{inter\text{-}individual} + CV^2_{method} + CV^2_{handling} (3)$$

Methodological and handling variabilities were assessed in separate

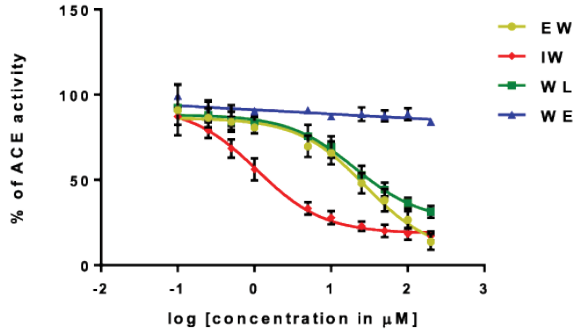

Figure 1: Concentration-response curves for inhibitory effects of tryptophan containing peptides glutamic acid-tryptophan (EW), isoleucine-tryptophan (IW), tryptophan-leucine (WL) and tryptophan-glutamic acid (WE) using rabbit lung ACE. Experiments reveal concentration dependent reductions in ACE activity with IW, EW and WL. In contrast, WE did not show any significant ACE inhibiting potency, n=3.

	Human plasma ACE	Rabbit lung ACE	Human recombinant ACE
IW	38.8 ± 3.15 µM	1.9 ± 0.6 µM	21.4 ± 5.2 µM
EW	121.7 ± 7.8 µM	26.2 ± 4.2 µM	106.5 ± 16.6 µM
WL	81.6 ± 6.3 µM	41.4 ± 6.2 µM	85.3 ± 12.1 µM
Captopril	11.9 ± 0.6 nM	3.8 ± 0.3 nM	11.4 ± 4.5 nM

Table 1: IC_{50} values for tryptophan-containing dipeptides and captopril on ACE activity. Data given as mean values ± SD, n=5-10.

experiments and found to be small (0.5 and 6.6%, respectively; Table 2). Knowledge of these variabilities then permitted to calculate true inter-individual variability

$$CV_{\text{inter- individual}} = (CV^2_{\text{observed}} - CV^2_{\text{method}} - CV^2_{\text{handling}})^{1/2} (4)$$

The inter individual variability was calculated to be 55.9 % and, thus, accounting for most of the observed variability (56.3%). If stability of IW differs between volunteers, this should be evident from plasma concentrations after adding IW. As seen in Figure 3A and B the final concentration of exogenously added IW after 1 hour of incubation in plasma decreased to various extents between plasma of different volunteers. Figure 3C shows the RP-HPLC chromatogram of IW and tryptophan (W) after incubation of IW for 1 hour in plasma samples of two volunteers. During incubation IW was degraded to the amino acids isoleucine and tryptophan. The respective chromatograms showed a decrease of the IW peak (retention time 8.8 min) and a simultaneous increase of the hydrolysis product tryptophan (retention time 8 min).

Figure 3A clearly indicates that the stability of IW in human plasma differs between volunteers. Thus, we addressed the question whether inhibition of plasma protease(s) might reduce this inter-individual variability. In the first step, the possibility of involvement of human plasma ACE in the degradation process was studied. IW was incubated with recombinant human ACE for one hour, and it was found that there was no significant difference in comparison to IW incubated in PBS (Figure 4A). Thus, ACE did not account for the inter-individual variability of the plasma half-life of IW. In a second step the involvement of other plasma proteases in the degradation process was

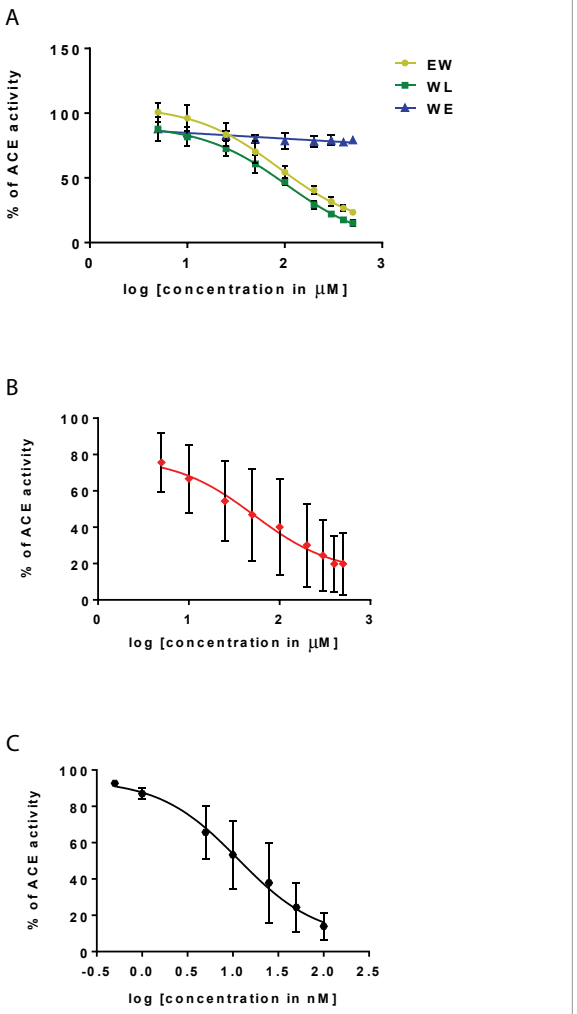

Figure 2: (A) Concentration-response curves for EW, WL, and WE on human plasma ACE activity. Only EW and WL showed concentration dependent reductions of ACE activity, n=5. (B) Concentration-response curve for IW on ACE activity. Despite prominent reduction of ACE activity there is a large variability of ACE inhibition as reflected by the high standard deviation, This variability was not observed with other peptides as shown in panel A. (C) Concentration-response curve for captopril, n=5.

	$CV_{Observed}$	$CV_{Inter-individual}$	CV_{Method}	$CV_{Handling}$
IW	56.3 ± 5.3	55.9 ± 5.3	0.5 ± 0.2	6.6 ± 3.2
EW	14.6 ± 3	13 ± 3.3		
WL	15.3 ± 1.7	13.8 ± 1.9		
Captopril	32.6 ± 0.07	32.3 ± 0.6		

Table 2: Coefficients of variation of peptide and captopril concentrations measured. Coefficients of variation were calculated from the percentage ratio of SD over the mean value. Data given as mean values ± SD, n=3-10.

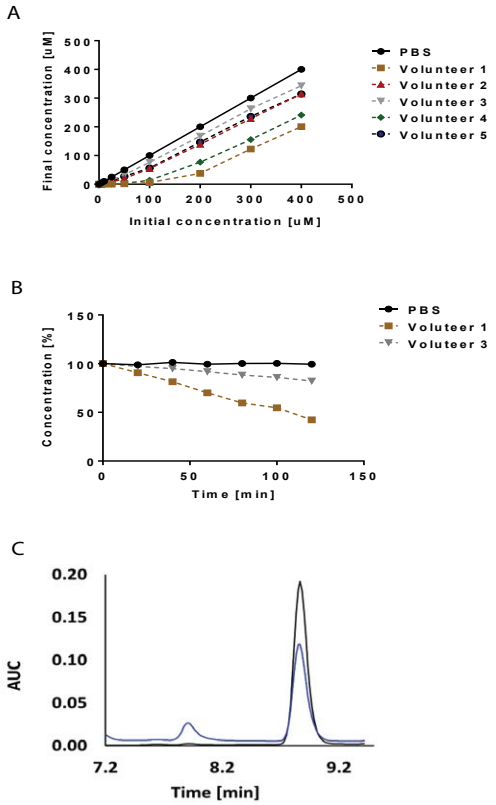

Figure 3: IW degradation in human plasma of different volunteers. (A) Final concentrations of IW are plotted versus the starting concentrations. Measurements of final concentrations were made one hour after adding IW to plasma samples, n=5. (B) Time dependent decrease of IW concentration in plasma of two volunteers with different plasma degradation rates. For comparison, stability of IW in PBS is shown. (C) Representative HPLC-chromatograms from two volunteers (curves in blue and black, respectively) used for quantification of IW and tryptophan (W) concentrations after one hour incubation in plasma. A higher degradation rate is reflected by the more pronounced decrease of the IW peak and a respective rise of the tryptophan peak. Thus, plasma of the volunteer encoded by the blue curve exerts a higher protease activity.

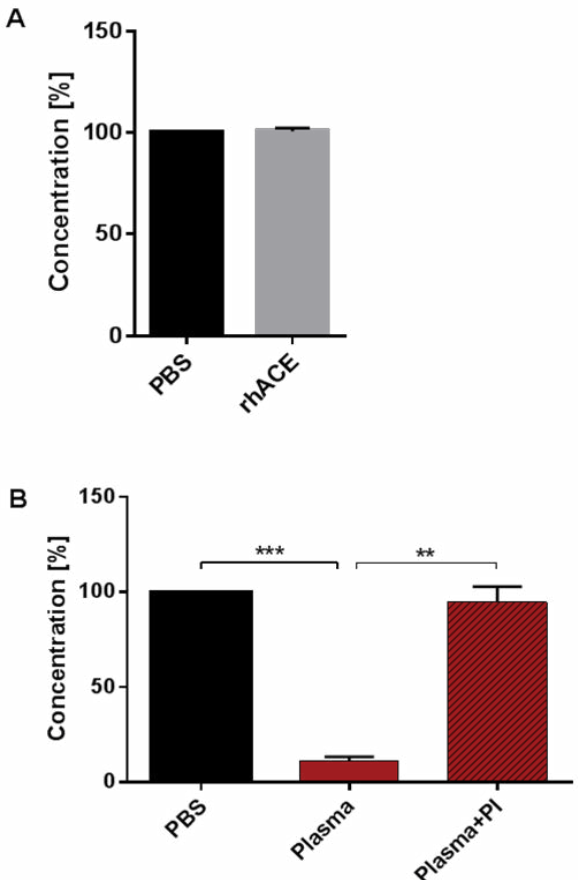

Figure 4: (A) Stability of IW during incubation with recombinant human ACE (rhACE). Recombinant human ACE did not reveal any effect on IW concentration. For comparison IW incubation in PBS is shown, n=3. (B) Degradation of IW in human plasma and effect of protease inhibition. IW was incubated in PBS, plasma and plasma plus protease inhibitor mixture (PI), respectively. IW was well preserved in plasma in presence of the protease inhibitor cocktail. ** p<0.01 versus PBS, *** p<0.001 versus plasma, n=3.

tested. A protease inhibitor cocktail was added to the plasma and IW concentrations measured after 1 hour of incubation showed a significant increase in comparison with the concentration of IW in plasma without protease inhibitor cocktail (Figure 4B).

The difference in plasma peptide stability was expected to affect the apparent inhibitory potency of IW. To avoid this, peptides were tested against recombinant human ACE activity (Figure 5). As shown in table 1 this did not change the rank order of peptides with respect to their plasma ACE inhibition efficacy.

Discussion

Several studies suggest that specific peptides hydrolyzed from milk protein may lower arterial blood pressure not only in hypertensive animal models but also in hypertensive humans [9,10]. However, although effective clinical trials have been reported, others did not find significant effects of whey peptides in hypertensive patients [11]. In addition to the dosage of the natural peptides and their absorption from complex food products, the *in vivo* fate of resorbed peptides is important when addressing in vivo efficacy. Therefore, in this study we investigated the effects of tryptophan containing dipeptides on human ACE activity as well as their stability in human plasma.

The ACE inhibitory effect of IW was previously tested using rabbit lung ACE and found to be the highest among peptides isolated from whey protein [12]. The present study also addressed the ACE inhibitory potential of other tryptophan containing peptides e.g. EW, WE and WL (Figure 1, Table 1). IC_{50} values for EW and WL were found to be 13- to 20- fold higher than that for IW when tested on rabbit lung ACE. In contrast, WE did not inhibit rabbit lung ACE activity. It should be noted that even dipeptides with the highest ACE inhibiting efficacy had a much lower efficacy as compared with captopril.

Rabbit lung ACE differs from human ACE with respect to the amino acid sequence [13]. It is thus to be expected that IC_{50} values may differ for the two species. Therefore, all dipeptides were tested against human plasma ACE activity. Except for WE all peptides caused concentration dependent reductions in ACE activity. The rank order of IC_{50} values was IW, WL, and EW. With respect to WL and EW this rank order differs from that obtained in rabbit lung, although the difference is small. Conversely, a prominent difference in the IC_{50} values for all peptides is observed versus that of captopril in all models tested (Table 1).

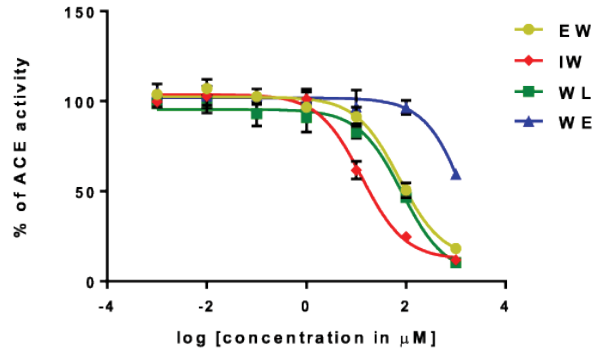

Figure 5: Concentration-response curves for whey peptides on recombinant human ACE activity. All peptides showed a concentration dependent reduction of ACE activity. The highest potency had IW followed by EW and WL. WE had only a minor inhibitory activity at considerably higher concentrations, n=3.

The salient feature for human plasma ACE inhibition with IW and captopril was the observed high standard deviation of plasma ACE activity. Assessment of various independent factors which contribute to overall variability, i.e. inter-individual, temporal, handling and methodological variability proved that handling and methodological variability were very small and could not explain the degree of observed variability. Also, ACE activity was temporally stable in the same individual (i.e. no temporal variability) which is in agreement with Alhenc-Gelas et al. [8]. Hence, the observed variability must be largely attributed to inter-individual differences in the efficacy of IW to inhibit plasma ACE. Interestingly, this inter-individual variability was remarkably smaller for EW, WL and WE (Figure 2).

The inter-individual variability was likely due to differences in IW stability in the plasma of different volunteers. Hence, we assessed IW and W concentrations in plasma of different volunteers following addition of defined quantities of IW. As shown in Figure 3B there was a remarkable difference in the recovery of IW after defined periods of time and the loss in IW was largely reflected by a respective rise in the W concentration (Figure 3C). Because the specific peptidase(s) which may account for hydrolysis of IW is (are) unknown to date, we tested whether application of a protease inhibitor cocktail enhanced the stability of IW. This revealed to be the case (Figure 4B). Although EW and WL did not exhibit a large inter-individual variability (Table 2), these peptides exhibited an even higher stability in human plasma after addition of a protease inhibitor cocktail. Meanwhile, WE was very stable in the plasma and adding protease inhibitor cocktail did not affect its concentration (data not shown). While ACE activity was tested to be temporally stable, which is in accordance with literature data [8], it may be of interest to note that IW stability also showed a temporal intra-individual variability in plasma samples obtained from a single volunteer on three successive days (data not shown). This in turn could affect the inter-individual variability of ACE inhibition. Therefore, future studies should clarify peptidase identity responsible for hydrolysis of tryptophan containing peptides and their inter-individual and temporal variability in human plasma. In first experiments we observed that peptidase activity is probably Ca^{2+}-dependent and largely brought about by an aminopeptidase, because it is inhibited with EDTA and bestatin (data not shown).

In addition to IW, we observed a clear heterogeneity of ACE inhibiting efficacy of captopril in plasma of different volunteers. Because captopril is not affected by proteases [14], the reason for this variability must differ from that of dipeptides. Inter-individual variability of the efficacy of pharmaceutical ACE inhibitors, e.g. captopril, has been reported in the literature. Likely, ACE polymorphism is involved here [15,16]. Others have suggested differences in drug metabolizing enzymes [17].

To avoid the breakdown effect of plasma proteases which affected the peptide concentration and, hence, the IC_{50} calculations, all peptides were tested against recombinant human ACE activity. Again, IW showed the highest potency, and other peptides were ranked WL, EW, WE (Figure 5). Thus, these measurements reconfirm the rank order of potency as assessed in human plasma (Figure 2). It may then be concluded that the high efficacy of IW was modulated by plasma protease activity. Such protease activities, however, do not seem to reverse the rank order of potency of tryptophan containing peptides.

In conclusion, when considering tryptophan containing whey peptides as food additives with the goal of ACE inhibition, in addition to their direct effect on ACE, their plasma stability should be considered. Obviously, plasma protease activity differs remarkably between individuals. For effective ACE inhibition by whey peptides a mixture of various peptides, e.g. IW, EW and WL may be considered to avoid inefficacy in a certain fraction of individuals due to high activity of plasma proteases.

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