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Angiotensin Converting Enzyme Inhibitory Peptides from a Lactotripeptide-Enriched Milk Beverage Are Absorbed Intact into the Circulation[1]

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Abstract

Food products containing angiotensin converting enzyme (ACE) inhibitory peptides reportedly play a role in treatment of mild hypertension. The aim of this placebo-controlled crossover study was to assess the bioavailability of Ile-Pro-Pro and 7 other ACE-inhibiting peptides present in a lactotripeptide (LTP)-enriched yogurt beverage and whether meal intake affects Ile-Pro-Pro bioavailability. Six male and female subjects randomly consumed an LTP-enriched yogurt beverage or a placebo in the fasted state and an LTP-enriched yogurt beverage in the fed or fasted state. The area under the curve (*AUC*) of Ile-Pro-Pro after the LTP treatment in the fasted state was 2.1-fold of that after the placebo treatment ($P < 0.001$). The maximum peptide plasma concentration (C_{max}) value was greater after consumption of the LTP-enriched beverage (897 ± 157 pmol/L) than after the placebo treatment (555 ± 0.09 pmol/L; $P < 0.001$) with a greater time after ingestion when reaching C_{max} (T_{max}) in the placebo treatment. Plasma concentrations of the peptides Leu-Trp, Phe-Tyr, Ile-Tyr, and Leu-Pro-Pro increased compared with baseline ($P < 0.05$) in the LTP-enriched and placebo treatment when consumed in the fasted state. However, ΔC_{max} values differed significantly between the placebo and LTP-enriched treatment only for Leu-Pro-Pro. Meal intake affected Ile-Pro-Pro concentrations. When the beverage was consumed after a meal, the *AUC* of Ile-Pro-Pro was 1.3-fold ($P < 0.05$) of the *AUC* derived from premeal intake. This was due to an increase in the plasma elimination half-life ($P < 0.05$); C_{max} and T_{max} were not affected by meal intake. In summary, this is the first demonstration, to our knowledge, that the tripeptide Ile-Pro-Pro selectively escapes from intestinal degradation and reaches the circulation undegraded. J. Nutr. 137: 953–958, 2007.

Introduction

Bioactive peptides are increasingly becoming of interest in the development of functional food products, as selected food proteins contain precursor sequences of peptides that may exert a physiological effect in the body when released from the parent protein (1–3).

One of the best-studied classes of bioactive peptides so far is the class of peptides with blood pressure-lowering activity. To date, several studies in spontaneously hypertensive rats and humans with elevated blood pressure demonstrated a blood pressure-lowering effect of selected fermented or partly enzymatically hydrolyzed milk products (4–10). In most cases, the antihypertensive effect of those bioactive peptides was explained by their angiotensin converting enzyme [(ACE)[2] EC 3.4.15.1] inhibitory activity. ACE is 1 of the key enzymes in blood pressure regulation, because it generates the vasoconstrictor angiotensin-II and inactivates the vasodilator bradykinin (11). Numerous ACE inhibitory peptides, derived from milk protein fractions but also from other food proteins, were described in vitro and predicted to be effective antihypertensives in vivo (3,12–14). However, it is difficult to establish a direct relation between ACE inhibitory activity detected in these products in vitro and antihypertensive activity in vivo, because bioavailability of peptides is a major issue. On this account, it was suggested that selected, very effective in vitro ACE inhibitory peptide hydrolysates from α-casein failed to lower blood pressure in models of hypertension because of intestinal breakdown of the peptides (15).

[1] Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.

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[2] Abbreviations used: ACE, angiotensin converting enzyme; *AUC*, area under the curve; C_{max}, maximum peptide plasma concentration; LC-MRM-MS, liquid chromatography-multi reaction ion monitoring-mass spectrometry; LTP, lactotripeptide; T_{max}, time after ingestion when reaching C_{max}; $t_{1/2}$, time in linear elimination phase in which peptide plasma concentration is halved.

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The underlying motivation for the research reported here is based on the assumption that crossing the peptidolytic barrier of the gastrointestinal tract is a prerequisite before any antihypertensive effect, whether via inhibition of ACE or other mechanisms, can be postulated, given that the bioactive peptides have to reach the cardiovascular system intact. However, whole peptide absorption was shown to be generally negligible (16,17), because the peptides have to resist the effective hydrolysis by gastric and pancreatic proteases as well as brush border amino- and carboxypeptidases, although a few exceptions were reported (18). Dipeptides and tripeptides are efficiently taken up by the enterocyte via peptide transporter 1(19), but the effective degradation by cytosolic and plasma peptidases limits their bioavailability. Surprisingly, studies on bioactive peptides have generally failed to consider these aspects. Only a few simulated gastrointestinal digestion studies were performed and demonstrated that, depending on the amino acid sequence, some peptides, particularly C-terminal Pro- and Pro-Pro-containing peptides, were stable under simulated gastrointestinal conditions, making these peptides more likely to be effective antihypertensives (20). However, in vitro absorption studies with in vitro ACE inhibitory peptides, stable under gastrointestinal conditions, showed very limited transport rates of peptides (21–23), implying only limited transport across the intestinal barrier of nonhydrolyzed peptides in vivo.

To our knowledge, only 2 bioavailability studies with antihypertensive, food-derived peptides were performed in vivo in human subjects (24,25). A single oral administration of an aqueous solution containing the peptide Val-Tyr led to a dose-dependent increase of Val-Tyr up to nanomolar concentrations in the plasma of normotensive as well as mildly hypertensive subjects. (24,25). Beside these 2 studies, bioavailability of food-derived blood pressure-lowering peptides has not been investigated in human subjects, to our knowledge.

The aim of this study was to characterize whether the blood pressure-lowering tripeptide Ile-Pro-Pro (8) derived from enzymatically hydrolyzed casein is bioavailable in humans. Furthermore, we wanted to investigate whether Ile-Pro-Pro and other ACE inhibitory peptides encrypted in milk protein are liberated in the intestinal tract and absorbed partly in a nonhydrolyzed form. Therefore, we examined in healthy male and female subjects a detailed kinetic profiling of Ile-Pro-Pro after oral consumption of a lactotripeptide (LTP)-enriched yogurt beverage and determined plasma concentrations of 7 other peptides (Leu-Pro-Pro, Ala-Trp, Ile-Trp, Leu-Trp, Val-Tyr, Ile-Tyr, and Phe-Tyr) with known high, in vitro ACE inhibitory activities (26). In addition, we gave special attention to the fact that consumption of the LTP-enriched beverage together with a meal could affect Ile-Pro-Pro bioavailability. Thus, we investigated the effect of consuming the beverage either in a fasted state, i.e. in the morning 30 min before breakfast on an empty stomach, vs. a fed state, i.e 30 min after the start of breakfast.

Materials and Methods

Subjects. Participants aged 18–55 y were recruited from inhabitants of the town of Vlaardingen and surroundings. Subjects were screened to be generally healthy as assessed by means of a screening questionnaire, BMI assessment (19–27 kg $\cdot$ m^{-2}), standard hematology, and urine. The subjects were all nonsmokers and had not donated blood at least 4 wk (men) or 8 wk (women) before the start of the study.

A total of 17 subjects were screened and 6 subjects plus 3 reserve subjects were included in the study. Three persons withdrew from the study for personal and medical reasons; these subjects were replaced by the 3 reserve subjects. The 6 subjects (4 female, 2 male) were randomly assigned to 1 of the 6 treatment sequences (see "Study design"). Participants were informed about the study and all subjects signed an informed consent form before participation. The Medical Ethical Committee of Wageningen University approved the study (approved November 2005). The baseline characteristics of study participants are summarized in **Table 1**.

Study design. The study was a randomized, placebo-controlled, full crossover intervention study performed at the Unilever Food and Health Research Institute, Vlaardingen, The Netherlands. The study was conducted following the guidelines of Good Clinical Practice. The study consisted of 3 intervention days, which were separated by 2 6-d washout periods. In the morning of each intervention day, the subjects who had fasted overnight came to the study facility and received 1 intervention. The subjects were randomly allocated to 1 of the 6 possible intervention sequences. In intervention 1, subjects consumed a 250-mL beverage containing 57 mg of LTP (20.4 mg Ile-Pro-Pro, 20.0 mg Val-Pro-Pro, and 16.5 mg Leu-Pro-Pro, subsequently referred to as "LTP-enriched" test beverage) after a 10-h overnight fast and 30 min prior to intake of a standardized breakfast. In intervention 2, subjects consumed an identical beverage 30 min after the start of a standardized breakfast. In intervention 3, the control intervention, a 250-mL beverage without added LTP equivalents was consumed after a 10-h overnight fast and 30 min prior to intake of a standardized breakfast. The LTP-enriched yogurt beverage and the placebo beverage were similar with respect to their content in carbohydrate, fat, protein, and calcium content (**Table 2**). Blood was sampled directly before consumption of the beverage ($t = 0$) and at 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75, 90, and 120 min after consumption of the beverage. In intervention 2, we took an additional sample before consumption of the breakfast ($t = -30$ min). During the study, the subjects were encouraged to minimize changes in composition of their habitual diet. In addition to the 10-h fasting period, the subjects had a 2-d run-in period before each intervention day in which they were not allowed to consume fermented dairy products such as cheese and yogurt.

Standardized breakfast. On the intervention days, we gave subjects the LTP-enriched or placebo yogurt beverage and a standardized breakfast at the study facility. The standardized breakfast consisted of 2 slices of 70 g whole wheat bread, 10 g of spread, 30 g of strawberry jam, and 125 mL of semiskim milk. During the intervention days, subjects were allowed to drink a standardized amount of water. The breakfast provided 1.4 MJ energy, with 68, 14, and 18% of the energy derived from carbohydrate, protein, and fat, respectively. Subjects consumed the same standardized breakfast on all study days.

Study product. All test beverages were produced from pasteurized, acidified, semiskim yogurt. The LTP in the LTP-enriched yogurt beverages were derived from AmealPeptide powder (Calpis). AmealPeptide powder was manufactured by enzymatic hydrolysis of milk casein followed by spray drying. AmealPeptide contains high concentrations of the ACE-inhibiting tripeptides Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro. AmealPeptide powder was added to the semiskim yogurt at a concentration of 2.8%. An orange flavor concentrate was mixed with the yogurt and the mixture was pasteurized and poured aseptically into 250-mL plastic bottles. The placebo beverage was similar to the LTP-enriched beverage but did not contain AmealPeptide powder. The protein content was adjusted to the protein level in the LTP-enriched yogurt beverage by adding 2.8% whey protein isolate. The nutritional compositions of the LTP-enriched and the placebo yogurt beverage are given in Table 2.

Collection of plasma samples. We collected venous blood samples in chilled K_3-EDTA-treated tubes. Plasma was separated by centrifugation

TABLE 1 General characteristics of study participants[1]

Parameter	Male, $n = 2$	Female, $n = 4$
Age, *y*	21.0 ± 2.0	41.8 ± 7.1
Weight, *kg*	73.3 ± 1.1	67.4 ± 3.7
Height, *cm*	186.5 ± 7.3	169.0 ± 3.0
BMI, *kg/m^2*	21.0 ± 1.0	23.5 ± 0.6

[1] Data are means ± SD.

TABLE 2 Nutritional composition of the test beverages[1]

Nutritional content	LTP-enriched beverage	Placebo beverage
Energy, $kJ \cdot L^{-1}$	3349	3349
Carbohydrates, $g \cdot L^{-1}$	125	132
Glucose	13	13
Fructose	42	42
Sucrose	45	45
Lactose	23	23
Maltose	<0.5	<0.5
Protein, $g \cdot L^{-1}$	51	51
SMP[2]	23	23
LTP powder	28	—
WPI	—	28
LTP, $mg \cdot L^{-1}$		
Ile-Pro-Pro	81.6 ± 1.6	<0.5
Leu-Pro-Pro	66.0 ± 2.0	<0.5
Val-Pro-Pro	80.0 ± 1.2	<0.5
Fat, $g \cdot L^{-1}$	3.1	2.9
Ca^{2+}, $mg \cdot L^{-1}$	1030	1000
Mg^{2+}, $mg \cdot L^{-1}$	110	110
Na^{+}, $mg \cdot L^{-1}$	760	330
Lactic acid, $g \cdot L^{-1}$	6.6	6.1
Citric acid, $g \cdot L^{-1}$	2.9	29
Acetic acid, $g \cdot L^{-1}$	0.1	<0.1
Ash, $g \cdot L^{-1}$	7.3	6.4
Moisture, $g \cdot L^{-1}$	810	802

[1] Values are single measures, LTP content expressed as means ± SD, $n = 3$.
[2] SMP, skim milk protein; WPI, whey protein isolate.

(3000 × *g*; 15 min, 4°C) within 30 min of collection. Trifluoro acetic acid was added to a final concentration of 1% and samples were stored at −80°C until assayed.

Peptide analysis by on-line reversed phase liquid chromatography-multiple reaction ion monitoring-mass spectrometry. Plasma concentrations of Ile-Pro-Pro, Leu-Pro-Pro, Ala-Trp, Ile-Trp, Leu-Trp, Val-Tyr, Ile-Tyr, and Phe-Tyr were determined using an liquid chromatography-multiple reaction ion monitoring-mass spectrometry (LC-MRM-MS) method described by van Platerink et al. (27). Peptide separation was performed on a 150- × 2.1-mm ODS3 column (Varian) with a particle size of 5 μm. The gradient started at 100% 0.1% trifluoro acetic acid in Milli-Q water and ended at 70% 0.1% trifluoro acetic acid in acetonitrile. To support the ionization, a 70/30 mixture of propionic acid and propanol-2 was added postcolumn at a flow rate of 50 $\mu L \cdot min^{-1}$. All analyses were conducted on an Alliance 2795 HPLC (Waters) combined with a Quattro Premier triple quadrupole mass spectrometer from the same supplier. We used [U^{13}C]Ile-Pro-Pro (BioPeptide) as internal standard. Calibration standards varied between 3 nmol $\cdot L^{-1}$ and 6146 nmol $\cdot L^{-1}$ and the concentration of the internal standard was constant at 3073 nmol $\cdot L^{-1}$. The specificity and sensitivity of the described HPLC-MRM-MS method was recently demonstrated by van Platerink et al. (27) with limits of quantification between 31 pmol $\cdot L^{-1}$ (Ile-Pro-Pro, Leu-Pro-Pro, Ile-Trp, Leu-Trp, and Phe-Tyr) and 36 pmol $\cdot L^{-1}$ (Ala-Trp, Ile-Tyr, and Val-Tyr).

Calculations and statistics. The primary outcome in this study was the area under the plasma peptide concentration vs. time curve after the test treatment. The area under the curve (AUC) was calculated by the linear trapezoidal rule as follows:

$$AUC = \frac{1}{2}\sum_{i=1}^{n-1}(m_{i+1} - m_i)(H_{i+1} + H_i),$$

where m_i is the i^{th} minute, H_i is the i^{th} available peptide value, and n is the number of minutes. Peptide bioavailability was determined over the period from 0 to 120 min; all values were considered in the $AUC_{0–120}$ calculation. The secondary outcomes were C_{max}, i.e. the maximum peptide plasma concentration; T_{max}, i.e. the time after ingestion when reaching C_{max}; and $t_{1/2}$, i.e. the time in the linear elimination phase in which peptide plasma concentration is halved. C_{max} and T_{max} were directly derived from the experimental data, whereas $t_{1/2}$ was calculated from the data within the terminal elimination phase. The rate constant for elimination (λ) was estimated by log/linear interpolation by repeated regression using the last 4 nonzero concentrations, then the last 5 concentrations, etc. Data points prior to C_{max} were not used. For each regression, an adjusted R^2 was computed and the λ derived from the regression with the largest adjusted R^2 was used. By definition, the terminal half-life $t_{1/2}$ was calculated as follows:

$$t_{1/2} = \ln(2)/\lambda.$$

The statistical analysis was performed using the software SAS 9.1 (SAS Institute). Descriptive analyses consist of distribution statistics (number of available observations, mean, SD, and 95% [CI]) for continuous data. The effects of treatments on both primary and secondary outcomes were evaluated by means of ANOVA including treatment, period, and subject in the model. The 3 treatments were compared with each other using the multiple comparison Tukey tests that assured an overall α error of 5%. The difference between each treatment was estimated on the basis of adjusted means. We did not adjust for multiplicity due to testing multiple (secondary) variables. We expected the secondary analyses to support the primary results. All tests were 2-sided with a significance level of 5%.

Results

ACE inhibitory peptide composition of the test beverage. The amount of Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro present as free tripeptide was 20.4 mg, 20.0 mg, and 16.5 mg in 250-mL of the LTP-enriched yogurt beverage, respectively. The placebo product contained <0.13 mg of each peptide in 250 mL, demonstrating that the LTP-enriched beverage indeed contained a greater proportion of free Ile-Pro-Pro (Table 2).

Maximum Ile-Pro-Pro plasma concentration and time after bolus intake. The proportion of subjects that provided sufficient data to construct complete kinetic profiles using a 1-compartment model was <50% of the data set. This was mainly due to a biphasic and scattered absorption phase (individual data not shown). Thus, kinetic parameters were calculated from all subject data by noncompartmental analysis without modeling the data. For each of the parameters, we constructed dot plots to evaluate the presence of extreme outliers. For each parameter, all individual values were <3 SD from the mean and data from all subjects were included in the analysis.

The mean plasma concentrations of Ile-Pro-Pro changed after an oral bolus of 250 mL of an LTP-enriched yogurt (2.8% AmealPeptide) or placebo (2.8% whey protein isolate) beverage (**Fig. 1**). In both cases, subjects consumed the beverage 30 min before intake of the breakfast. After a 10-h overnight fast and before administration of the test beverages, Ile-Pro-Pro plasma concentrations were ~300 pmol L^{-1} and did not differ between the LTP-enriched and placebo treatments (**Table 3**). Consumption of the whey protein isolate-containing placebo beverage led to an increase ($P \leq 0.05$) in Ile-Pro-Pro plasma concentrations to a C_{max} of 555 ± 80 pmol $\cdot L^{-1}$ plasma compared with baseline concentrations. However, after consumption of the LTP-enriched beverage, the C_{max} value of Ile-Pro-Pro was 1.6-fold of that after the placebo treatment and the T_{max} tended to be shorter after consumption of the LTP-enriched beverage compared with the placebo beverage ($P = 0.09$)(Table 3).

Plasma bioavailability of Ile-Pro-Pro (net AUC) and curve progression. Because Ile-Pro-Pro was detectable at baseline in

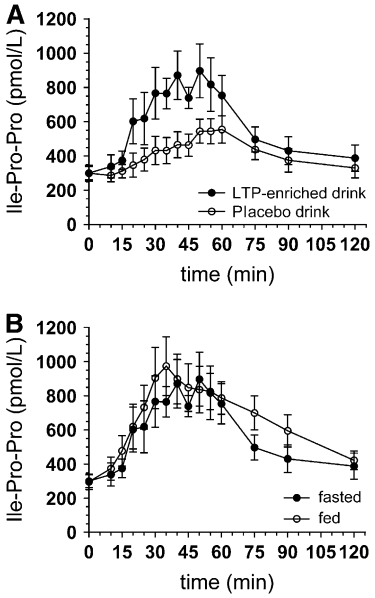

Figure 1 Plasma Ile-Pro-Pro concentrations in healthy subjects after consumption of LTP-enriched and placebo yogurt beverages in the fasted state (*A*) and after consumption of the LTP-enriched yogurt beverage in fasting and fed states (*B*). Values are means ± SD, $n = 6$.

all subjects, and because these levels varied between subjects, net *AUC* values for plasma Ile-Pro-Pro were calculated by correcting for baseline concentrations per subject. The net *AUC* for plasma Ile-Pro-Pro over a period of 120 min after subjects consumed the LTP-enriched yogurt beverage in the fasted state was 2.2-fold of that ($P \leq 0.001$) after consuming the placebo yogurt beverage (Table 3). The kinetic curves (Fig. 1*A*) showed that when subjects consumed the LTP-enriched yogurt beverage, the plasma Ile-Pro-Pro concentration increased with almost no lag time and differed from baseline ($P \leq 0.05$) 15 min after ingestion, whereas after the placebo treatment, the concentrations differed from baseline after 25 min ($P \leq 0.05$). After consuming both beverages, the IPP concentration returned to values near baseline by 120 min.

TABLE 3 Ile-Pro-Pro kinetic measurements in healthy subjects after consumption of an LTP-enriched and placebo yogurt beverage in the fed and fasted state[1]

	Treatment		
	Placebo fasted	LTP-enriched fasted	LTP-enriched fed
Dose[1] Ile-Pro-Pro			
$mg \cdot L^{-1}$	$<0.15^a$	81.8 ± 1.5^b	81.6 ± 1.6^b
$\mu mol \cdot L^{-1}$	$<1.50^a$	100 ± 12^b	100 ± 11^b
Baseline, $pmol \cdot L^{-1}$	299 ± 45	300 ± 38	296 ± 65
	(157−436)	(175−428)	(174−467)
C_{max}, $pmol \cdot L^{-1}$	555 ± 80^a	897 ± 157^b	973 ± 180^b
	(344−845)	(631−1663)	(636−1514)
T_{max}, *min*	54.2 ± 7.9	39.9 ± 9.9	41.7 ± 15.7
	(40−60)	(25−50)	(20−60)
AUC_{0-120}, $nmol \cdot min^{-1} \cdot L^{-1}$	18.0 ± 5.7^a	38.5 ± 19.9^b	47.5 ± 17.5^c
	(8.9−26.0)	(14.6−72.3)	(27.7−77.4)
HL[2], *min*	nd	26.4 ± 15.1^a	38.6 ± 13.5^b
		(5.7−44.1)	(28.7−58.8)
MRT, *min*	61 ± 4	52 ± 5	58 ± 5
	(56 ± 66)	(44 ± 60)	(49 ± 66)

[1] Values are expressed as LS means ± SD (range), $n = 6$. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.
[2] HL, Plasma half-life; MRT, mean residence time; nd, not determined.

Plasma concentrations of other ACE inhibitory peptides. Besides Ile-Pro-Pro, we determined plasma concentrations of the ACE inhibitory peptides Leu-Pro-Pro, Ala-Trp, Ile-Trp, Val-Tyr, Ile-Tyr, Phe-Tyr, and Leu-Trp in response to oral intake of an LTP-enriched and a placebo yogurt beverage in the fasted state. Likewise, background levels of these peptides were present in varying amounts in plasma of fasting subjects (data available in **Supplemental Table 1**). Especially the dipeptides Leu-Trp, Ile-Trp, and Ile-Tyr showed much higher background levels in human plasma compared with the tripeptides. Plasma levels of all peptides increased in response to intake of the LTP-enriched and the placebo yogurt beverage. The increase measured as ΔC_{max}, i.e. the difference between C_{max} and baseline concentrations, differed ($P \leq 0.05$) after the placebo ($96 \pm 34 \cdot$ pmol L^{-1}) and LTP-enriched (152 ± 85 pmol $\cdot L^{-1}$) treatments only for the tripeptide, Leu-Pro-Pro.

Effect of meal intake on IPP bioavailability: comparison of fasted and fed state. To test whether concomitant food intake can affect Ile-Pro-Pro bioavailability, subjects consumed the LTP-enriched beverage in fasted and fed states, i.e. 30 min before and 30 min after starting to eat a breakfast. All subjects consumed breakfast in <15 min. Intake of the LTP-enriched yogurt beverage shortly after a meal affected Ile-Pro-Pro bioavailability as judged from net *AUC* values. When the LTP-containing beverage was consumed in the fasted state, mean plasma *AUC* values were 1.2-fold of that after the placebo treatment (Table 3). Net *AUC* differed between the treatments ($P \leq 0.05$) (Table 3). The plasma half-life of Ile-Pro-Pro was prolonged ($P \leq 0.01$) when the LTP-enriched yogurt beverage was consumed after the breakfast (Fig. 1; Table 3). The C_{max} and T_{max} values did not differ between the fed and fasted treatment ($P > 0.05$).

Discussion

Uptake of intact peptides from the gut lumen into the circulatory system is controversial. Here, using a highly sensitive and specific LC-MRM-MS method, we described for the first time, to our knowledge, that the tripeptide Ile-Pro-Pro was present in plasma of human subjects and that its plasma levels increased after intake of an LTP-enriched yogurt beverage. Furthermore, we showed that Ile-Pro-Pro plasma concentrations were augmented by concomitant intake of the LTP-beverage and a meal.

Our double blind, placebo-controlled crossover study strongly suggests, although does not directly demonstrate, that Ile-Pro-Pro is bioavailable after oral consumption of an LTP-enriched yogurt beverage. Evidence for this was provided by the net *AUC* and C_{max} data for Ile-Pro-Pro that were significantly higher for the LTP-enriched yogurt beverage compared with the placebo, in which the LTP powder was replaced with Ile-Pro-Pro-free whey protein isolate. In addition, ingesting the LTP-enriched beverage significantly increased plasma concentrations of the ACE inhibitory peptide Leu-Pro-Pro compared with the placebo. The increased plasma concentrations of Ile-Pro-Pro detected in our study were similar to those of the dipeptide Val-Tyr detected in plasma after oral dosing of 6 mg and 12 mg Val-Tyr (24,25). In contrast to the long half-life of 3.1 h of Val-Tyr detected (24,25), Ile-Pro-Pro was cleared from plasma much faster with an elimination half-life of ~30 min. A 3rd study,

reporting a plasma half-life of 20 min for the dipeptide Ala-Gln after continuous intravenous infusion (28), supports our findings of rapid disappearance of peptides from plasma. In addition, the C_{max} of Ile-Pro-Pro was ~1 nmol · L^{-1}, which is far below its effective concentration on ACE inhibition determined in vitro (50% inhibition constant = 5 μmol · L^{-1}) (29), although higher doses of Ile-Pro-Pro were used in our study than in human studies showing a blood pressure-lowering effect in response to LTP consumption (30,31). Thus, it remains questionable whether those peptides lower blood pressure solely via an ACE-inhibiting mechanism.

Surprisingly, plasma Ile-Pro-Pro concentrations were also significantly elevated above baseline concentrations after consumption of the whey protein isolate-containing placebo. This suggests that Ile-Pro-Pro was generated in the intestinal tract by luminal and brush border peptidases, as the placebo beverage contained no free Ile-Pro-Pro. Minor amounts of Ile-Pro-Pro-precursors were present in the yogurt matrix, e.g. κ-casein, and in the milk protein consumed with the breakfast. The lagged increase in plasma concentration of Ile-Pro-Pro and the trend of higher T_{max} values after the placebo treatment (54.2 ± 7.4 min) than after the LTP-enriched treatment (39.9 ± 9.9 min) (P = 0.09) suggest that Ile-Pro-Pro after the placebo treatment was indeed generated during the digestion process in the intestinal tract. Evidence for the release of Ile-Pro-Pro from large peptide sequences in the intestinal tract was given in in vitro digestion studies using simulated gastrointestinal fluids, which showed liberation of Ile-Pro-Pro from casein peptides (32,33). The difference in net *AUC* of Ile-Pro-Pro between placebo and LTP-enriched treatment was small, although significant, compared with their large concentration difference in the test beverages. This raises the question whether hydrolysis of Ile-Pro-Pro-containing protein sources prior to consumption is necessary to increase the bioavailability of bioactive peptides. This question needs to be addressed in a separate study in which the bioavailability of Ile-Pro-Pro is directly compared between nonhydrolyzed casein sources and hydrolyzed casein.

The net *AUC* value of Ile-Pro-Pro ($P \leq 0.05$) was larger after consumption of the LTP-enriched beverage in the fed compared with the fasted state. Furthermore, the elimination half-life of Ile-Pro-Pro was increased ($P \leq 0.01$) after the fed treatment. A possible mechanism causing this effect could be that meal proteins act in an unspecific, competitive manner with the tripeptide for luminal, brush border, and cytosolic peptidase activity and thus protect Ile-Pro-Pro from hydrolysis by saturation of peptidases. Furthermore, meal-derived peptidase inhibitors, e.g. antitrypsin and antichymotrypsin activity as described in wheat (34), could cause the increase in Ile-Pro-Pro plasma concentrations.

We found that a variety of dipeptides, including the dipeptide Val-Tyr, were present after a 10-h fasting period in human plasma in pico- and nanomolar concentrations, as determined by means of LC-MRM-MS. To our knowledge, this is the first demonstration of the existence of endogenous di- and tripeptides in human plasma. This is in contrast to the observation of Matsui et al. (25), who showed the absence of the dipeptide Val-Tyr in plasma of fasting subjects. An explanation for the differences might be the analytical method used. However, it is difficult to predict from the results of our study what the origin of Ile-Pro-Pro, Leu-Pro-Pro, and the other dipeptides in plasma at baseline is. Given the short half-life of Ile-Pro-Pro of ~30 min determined in this study, it is unlikely that the Ile-Pro-Pro detected before intake of the test beverages is derived from dietary origin. On the other hand, numerous human proteins contain the Ile-Pro-Pro sequence, e.g. heparanase and cadherin-14 (35,36), suggesting that the peptides detected at baseline could be derived from the constant turnover of proteins that contain the respective sequences. The fast attainment of peak plasma concentrations in <1 h for Ile-Pro-Pro and the differences in net *AUC* between LTP-enriched and placebo treatment strongly suggests that those peptides are derived from the test beverages. Secretion of endogenous peptides in response to meal intake, however, should be considered. After both treatments, significantly increased plasma levels of Leu-Trp, Phe-Tyr, and Ile-Tyr were detected after intake of the test beverages. Because the LTP-containing beverage was not specifically enriched in dipeptides, no difference between treatments was detected. Interestingly, the levels of dipeptides in plasma tended to be generally higher than those of the tripeptides. This is in line with the observation that bioavailability of peptides increases with decreasing chain length (37).

The low plasma concentrations of Ile-Pro-Pro observed in this study confirm the low oral bioavailability of peptides and indicate that presystemic enzymatic degradation and poor penetration of the intestinal barrier determines this low bioavailability. In general, permeation of solutes across the gastrointestinal epithelium occurs via paracellular and transcellular absorption pathways and can be influenced by active and polarized transport systems. Di- and tripeptides are taken up actively and quickly by enterocytes via peptide transporter 1. The efflux of peptides into the blood, however, is limited, because no active transport system at the basolateral side of the enterocyte supports their release (19,38). The large variation in physicochemical properties (size, charge, lipophilicity) of peptides, which determines their permeability, makes it impossible to draw general conclusions about their potential to cross the intestinal barrier via paracellular transport or passive diffusion (39). Ile-Pro-Pro and Leu-Pro-Pro, however, are hydrophilic peptides with logP values < -2.2. Thus, those peptides are not expected to follow a transcellular pathway of absorption through passive diffusion (40). The size of the paracellular space of 10–50 Å in the duodenum and jejunum would allow a limited paracellular flux of those peptides and may account for the low plasma concentrations detected in the present study. In vitro transport studies across Caco-2 monolayers showing very low permeability constants for Val-Val, Val-Pro-Pro, and Gly-Gly-Tyr-Arg support this (21–23). Furthermore, peptides consisting of proteinogenic amino acids were rapidly hydrolyzed by apical and cytosolic peptidases in the Caco-2 system (23), indicating that the metabolic instability is a major factor in limiting peptide bioavailability.

In summary, in humans, the peptides Ile-Pro-Pro and Leu-Pro-Pro were detected in plasma in picomolar concentrations. Moreover, plasma concentrations increased significantly to nanomolar concentrations when ingested in the form of an LTP-enriched yogurt beverage. In addition, concomitant intake of the LTP-enriched yogurt and a meal further increased plasma concentrations of Ile-Pro-Pro.

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