Transepithelial Transport and Stability in Blood Serum of Angiotensin-I-Converting Enzyme Inhibitory Dipeptides

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The dipeptides Ala-Trp, Val-Phe, and Val-Tyr inhibit the angiotensin-I-converting enzyme. They are encrypted within the primary sequences of different food proteins, e.g. milk proteins. The angiotensin-I-converting enzyme inhibitory potency of these synthetic dipeptides was quantified using a spectrophotometric assay. The dipeptides showed no adverse effects on differentiated Caco-2 cells (model for human intestinal epithelium), as confirmed by transepithelial electrical resistance, microscopy and the activity of the brush-border enzyme dipeptidyl aminopeptidase IV. Furthermore, the transport of these bioactive dipeptides through intact Caco-2 monolayers and their stability to incubation in human blood serum has been demonstrated for the first time. Low molecular mass peptides represent the minimal structures required for angiotensin-I-converting enzyme inhibition which have a high potential bioavailability. Therefore, they may act as target peptides in enriched hydrolysates for the preparation of an angiotensin-I-converting enzyme inhibitory peptide and for the use in special formulations as functional foods/foods of specified health use.

Key words: Angiotensin Converting Enzyme (ACE), Bioactive Peptides, Transepithelial Peptide Transport

Introduction

Bioactive substances of food origin can be defined as components of food which may exert regulative activities in the human organism beyond basic nutrition. The existence of a large diversity of food-derived bioactive substances having a non-nutrient character can be considered as an aid in maintaining good health. These substances may be used as ingredients in functional foods/foods of specified health use (FOSHU) which can be consumed to reduce the risk of disease or to enhance certain physiological functions.

Milk proteins are precursors of different bioactive peptides having, e.g. antihypertensive, antithrombotic, opioid, antimicrobial, cytomodulatory, immunomodulatory or mineral binding effects (for review: Meisel and Schlimme, 1990; Meisel, 1997, 2005; Clare and Swaisgood, 2000). The biological activities encrypted in major milk or other food proteins are latent until their release or activation by enzymatic proteolysis, e.g. during gastrointestinal digestion or food processing (Meisel, 2001; Meisel and Bockelmann, 1999).

Food proteins, especially from milk, are precursors for a range of peptides which inhibit the angiotensin-I-converting enzyme (ACE) (for review: Meisel et al., 2006). Casein-derived inhibitors of ACE are known as casokinins (Meisel, 1993), whereas whey-derived inhibitors are known as lactokinins (FitzGerald and Meisel, 1999). Most antihypertensive peptides have an inhibitory effect on ACE which is a key enzyme in the regulation of peripheral blood pressure and electrolyte homeostasis. This enzyme converts angiotensin I to the highly potent vasoconstrictor angiotensin II (Skeggs et al., 1956) and inactivates the vasodilatory peptide bradykinin (Erdös, 1999). Furthermore, ACE plays a key physiological role in the regulation of the neuroendocrine and immune systems due to its wide substrate specificity. ACE is a membrane-anchored dipeptide-liberating carboxypeptidase (peptidyl dipeptide hydrolase, EC 3.4.15.1) which consists of two homologous domains (N and C domain). Each domain contains an active site which catalyzes the hydrolysis of angiotensin I (Inagami, 1992). The C domain seems to be necessary for controlling blood pressure (Natesh et al., 2003). It is worth noting that some peptides are effective ACE-inhibitors in vitro but
show no effect on blood pressure \textit{in vivo}. Some peptides may be susceptible to degradation by gastrointestinal enzymes, blood serum, and intracellular peptidases, respectively, or even to modification in the liver. The bioactive potential of di- and tripeptides is particularly high since small peptides can be absorbed in the intestine without being decomposed by digestive enzymes and thus reach target sites in the body following transport in the blood system. This has been confirmed by the results of several studies with mildly human hypertensive subjects (for review: Meisel et al., 2006), which definitively demonstrate a significant reduction in blood pressure following daily ingestion of food-derived ACE-inhibitors (e.g. Ile-Pro-Pro, Val-Pro-Pro). The hypotensive peptides used for human trials were obtained from milk protein hydrolysates (casein, whey protein), fermented milk and fish hydrolysates (sardine, dried bluefish/bo-nito) and were administered mostly as hydrolysates or as ingredients of sour milk per se. The dose of the main ACE-inhibitory peptides, which were not present in the placebo drink and thus are considered to be the active substances, was in the range of 2 to 6 mg. The oral ingestion of such relatively low amounts of these peptides may lower blood pressure to an extent which is comparable to a pharmacological treatment. However, the advantage of consuming food protein-derived peptides relates to the fact that no side effects and no significant changes in blood pressure of normoten-sive subjects have been observed (Meisel et al., 2006). These peptides can only lower blood pressure \textit{in vivo} following absorption across the intestinal epithelium and subsequent transport to their target sites. Therefore, it is proposed that determination of their ability to be transported across the intestines and their resistance to gastrointestinal and serum peptidases should be assessed prior to performing time-consuming and costly human studies.

Caco-2 cells provide a useful cell culture model of the human small intestinal epithelium, because these cells spontaneously develop enterocyte-like differentiation properties under standard culture conditions (Pinto et al., 1983; Hidalgo et al., 1989; Wilson et al., 1990; Audus et al., 1990; Shimizu and Dong, 2007). These include morphological polarity, formation of tight junctions and the expression of brush-border hydrolases and different transporters (Hidalgo and Borchardt, 1990; Shimizu, 1999; Rubio and Seiquier, 2002; Gnoth et al., 2002; Konishi and Shimizu, 2003). Moreover, the evaluation of potentially cytotoxic \textit{in vitro} effects of dipeptides is possible with different cytochemical methods according to the requirements stated in the EN ISO 10993-5 (1992). Accordingly, cytotoxicity is considered to include all adverse effects a test compound might have on the viability of cells, independently of the mode of action or the respective biochemical mechanism (Hartmann and Meisel, 2000).

To the knowledge of the authors, the potential intestinal transport of different bioactive peptides was demonstrated only in three \textit{in vitro} studies: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradyki-nin: vasodilator), Gly-Gly-Tyr-Arg (inhibitor of papain), Phe-Arg-Ala-Asp-His-Pro-Phe-Leu (ovo-kinin: ACE-inhibitor), Tyr-Pro-Phe-Pro-Gly (β-casomorphin-5: opioid peptide) (Shimizu et al., 1997), Val-Pro-Pro, Ile-Lys-Pro, Ile-Lys-Trp (Satake et al., 2002) and Ala-Leu-Pro-Met-His-Ile-Arg (ACE-inhibitors) (Vermeirssen et al., 2002).

The objectives of this \textit{in vitro} study were: to investigate the possible cytotoxic potential on differentiated Caco-2 cells of highly potent ACE-inhibitory dipeptides, to determine if they are transported through intact Caco-2 monolayers, and to determine their stability in human blood se-rum.

Materials and Methods

\textbf{Dipeptides, amino acids and ribonucleosides}

Ala-Trp, Val-Tyr, Val-Phe as well as phenylala-nine, tryptophan, tyrosine, and N6-dimethyladeno-sine (m6,2Ado), N6-(2-isopentenyl)-adenosine (ip-6Ado) were obtained from Bachem AG (Weil am Rhein, Germany), Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively.

\textbf{ACE-inhibition assay}

ACE-inhibitory activity was measured using a spectrophotometric assay based on the liberation of hippuric acid from hippuryl-l-histidyl-l-leucine (HHL) as catalyzed by ACE (Cushman and Cheung, 1971). ACE (8.3 mU; Fluka-Sigma-Al-drich, Buchs, Switzerland) and the synthetic HHL substrate (0.25 μmol; Fluka-Sigma-Aldrich) were incubated for 30 min at 37 °C with synthetic pep-tides; all components of the 0.25 mL assay mixtures were dissolved in 0.1 mol/L sodium borate.
buffer, 0.3 mol/L NaCl, pH 8.3. The liberated hippuric acid was extracted with ethyl acetate and then quantified at 228 nm (UV/VIS Spectrometer Lambda 40, Perkin Elmer GmbH, Überlingen, Germany). The amount of hippuric acid liberated under test conditions in the absence of an inhibitor was defined as 100% ACE activity. Furthermore, inhibition curves (% inhibition vs. log_{10} [μmol/L]) were constructed using at least seven separate analyses to calculate the concentration of test sample which inhibited 50% of the ACE activity (= IC_{50} value).

**Culture of Caco-2 cells**

Caco-2 cells (DSM ACC 169) were obtained from DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All cell culture materials were obtained from Sigma-Aldrich Chemie GmbH, unless indicated otherwise. Cells were routinely grown in 25 cm² plastic flasks (Corning®, Corning, NY, USA) at 37 °C in an atmosphere of 5% CO₂ (Hartmann et al., 2000). Cells were cultured in DMEM with 4.5 g/L glucose supplemented with 20% fetal calf serum, 4 mmol/L L-glutamine and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively). Caco-2 cells plated at 10⁵ cells/cm² were passaged every 5 to 6 days when confluency was reached; medium was changed every 2nd/3rd day. The Caco-2 monolayer was passaged using 0.05% trypsin/0.02% EDTA solution. Caco-2 cells were used between passage numbers 30 and 60. Caco-2 cells were seeded at a density of 10⁶/cm² in 12- and 6-well microtiterplates (MTP) (Costar®, Corning, NY, USA) on Transwell® inserts with polyethylene (PE) and polycarbonate (PC) membranes (por size: 0.4 μm). These membrane inserts separated an apical from a basolateral compartment in each well. Furthermore, cells were seeded at a density of 10⁴/200 μL in 96-well MTP with flat bottom (Costar®) to determine the activity of dipeptidyl aminopeptidase IV (DPP IV). All cells were cultured for 10 or 14 d under standard conditions (37 °C, 5% CO₂) with medium renewal every 2nd/3rd day.

**Transepithelial electrical resistance (TEER) measurement and microscopy to evaluate the enterocytic differentiation of Caco-2 cells**

Caco-2 cells (10⁵/cm²) were grown on PE membranes in 12-well MTP. Cells were cultured for 10 d (d₀ to d₁₀) under standard conditions (37 °C, 5% CO₂) and thus were allowed to differentiate. Peptide solutions (0.1 to 1.0 mmol/L) were applied to the apical compartment on the Caco-2 monolayers on day 10 (d₁₀). At the same time cells of 2 wells of each MTP were cultured in DMEM (negative control) as well as 1 mmol/L m6,2Ado (positive control). TEER was measured three times across the membrane and each cell monolayer on d₁₀ after incubation for 4 h and 24 h (d₁₀ + 4 h and d₁₀ + 24 h), respectively, using a Millicell ERS (Millipore Corporation, Billerica, MS, USA) device (Hartmann et al., 2000; Hashimoto et al., 1994) operated at room temperature. Mean values (MV) of TEER ± SEM of negative control on d₁₀ as well as after further 24 h of incubation with DMEM were in the range 409–596 Ω cm². After the measurement of monolayers incubated with dipeptides (test) a TEER index (I_{TEER} = MV_{test} / MV_{negative control}) ± SEM was calculated as mean of 3 replicates (where stimulation: > 1, inhibition: < 1). Only the positive control showed adverse effects on Caco-2 monolayers after 24 h of incubation: I_{TEER} in the range from (0.01 ± 0.01) to (0.05 ± 0.01) were calculated on d₁₀ + 24 h (MV of TEER: 6–21 Ω cm²). About 2 h after the TEER measurement all monolayers were observed by light microscopy (200-fold magnitude; Helmut Hund GmbH, Wetzlar, Germany) to evaluate potential alterations in cytomorphology.

**Determination of dipeptidyl aminopeptidase IV (DPP IV)**

This determination was established based on the method of Sanz and Toldrá (2001) and optimized to evaluate the enterocytic differentiation of Caco-2 cells. Cells (10⁴/200 μL) were grown in 96-well MTP and incubated under standard conditions for 14 d. On d₁₄ 100 μL medium were taken from each well and replaced by 100 μL dipeptide solution to obtain concentrations of 0.01, 0.1, and 1.0 mmol/L, respectively. Wells of negative or positive control were refilled with 100 μL medium or ip6Ado (1 mmol/L). Caco-2 monolayers were incubated for 4 and 24 h with test substance. Thereafter, all wells were twice decanted and washed with 200 μL 0.5 mmol/L tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.5; Bachem AG). Then 200 μL Ala-Pro-p-nitroanilide (0.5 mmol/L Tris-HCl buffer, pH 7.5; Bachem AG) were added to each well. After 30 min of incubation at 37 °C
Transport studies

Caco-2 cells (10^5/cm²) were grown on PC membranes in 6-well MTP and cultured for 14 d (d0 to d14) under standard conditions (37 °C, 5% CO₂) to obtain differentiated cells. The integrity of Caco-2 monolayers was evaluated by measuring the TEER at 37 °C on d14, directly before and 4 h after the transport study. Only intact Caco-2 monolayers (TEER > 200 Ω cm²) were used for transport studies. All selected Transwell® inserts were gently rinsed with Hanks’ balanced salt solution (HBSS) before 1.5 mL HBSS [pH adjusted to 6.0 with 2-morpholinoethanesulfonic acid (MES); Merck] were added on each monolayer, and the inserts were placed in a well containing 2.6 mL HBSS (pH 7.4). After equilibrating for 30 min at 37 °C, MES-HBSS was withdrawn from the apical side of the Caco-2 monolayer to be replaced by the test solutions, containing 4.0 mmol/L dipeptides (n = 2 for each dipeptide) or MES-HBSS which served as negative control. After further incubation for 60 min, the basolateral solutions were removed separately, stored at −24 °C, and later analyzed by RP-HPLC and an amino acid analyzer.

Incubation of dipeptides in human blood serum

Human blood was obtained freshly in-house from a healthy volunteer and stored at room temperature for 30–60 min. Serum was separated by centrifugation for 10 min at 280 × g and then stored in 1 mL aliquots at −80 °C. Freeze-dried dipeptide samples were dissolved in serum (~2 mg/mL) and incubated for 30 min at 37 °C. The sulfosalicylic acid (SSA, 5%, w/w; Merck)-soluble fractions obtained following incubation for 1 h at 5 °C and centrifugation for 3 min at 13,000 × g were used for reverse phase and gel permeation HPLC analyses after a storage at −24 °C.

Analytical and semi-preparative reverse phase HPLC (RP-HPLC)

The RP-HPLC-system consisted of two 422 Master pumps (Bio-Tek Kontron, Neufarn, Germany), two mixers M800 (Bio-Tek Kontron), an injector 7125 (Rheodyne, Bensheim, Germany), a column oven (self-produced), a diode array detector 440 (Bio-Tek Kontron), a Foxy fraction collector (Teledyne ISCO, Los Angeles, USA) and a data system KromaSystem 2000 (Bio-Tek Kontron). A LiChroCART Superspher 100 C18 end-capped 125 × 4 mm column, particle size 4 μm (Merck), was used for analytical as well as semi-preparative separations of samples of basolateral solution and of human serum. Freeze-dried and vacuum-evaporated samples (Speed Vac concentrator, Savant Instruments Inc., Farmingdale, NY, USA) were dissolved with 0.1% TFA in 70% (v/v) aqueous acetonitrile. Aliquots (150 μL) of sample solutions of synthetic dipeptides (~2 mg/mL), amino acids (~2 mg/mL), basolateral solutions and samples obtained following incubation in human serum were injected onto the RP-HPLC column. Selected samples of basolateral solutions were spiked with amino acids and dipeptides to identify the elution position of the corresponding test substance. Chromatography was performed at a flow rate of 1 mL/min. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in 50% aqueous acetonitrile. Both solvents were purged with helium. The gradient (I) was 5% B for 5 min and 5% B to 90% B in 85 min, followed by a decrease to 5% B within 5 min and further elution at this concentration for 15 min. For re-chromatography, a shallower gradient (II) of 5% B for 5 min and 5% B to 45% B in 85 min was used. Column temperature was 30 °C. Peaks were detected by absorbance at 205, 215 and 280 nm. To estimate the amount of peptides transported through Caco-2 monolayers, peptide calibration standards were prepared by diluting stock solutions of dipeptides to concentrations ranging from 0.001 to 4.0 mmol/L. The linear calibration functions (r ≥ 0.9955) and detection limits for Ala-Trp (1.2 μg/mL), Val-Phe (5.7 μg/mL) and Val-Tyr (8.0 μg/mL) were calculated according to DIN 32645 using Valoo® analytic software (Dr. Stella Wintermann, Bremen, Germany).
Analytical gel permeation HPLC

Gel permeation (GP) HPLC analyses were performed with the same system as used for RP-HPLC. A gel permeation column Superdex Peptide PE 7.5/300 (Amersham Biosciences, Freiburg, Germany) was used for analytical separations at room temperature. Aliquots (10 μL) of solutions of synthetic peptides (~2 mg/mL), amino acids (~2 mg/mL) and samples obtained after incubation in human serum were injected and eluted at a flow rate of 0.8 mL/min. The mobile phase used for isocratic elution was 30% acetonitrile in 0.1% TFA. Peaks were detected by absorbance at 210, 215 and 280 nm.

Amino acid analysis

Vacuum-evaporated samples (Speed Vac concentrator, Savant Instruments) which were collected after semi-preparative RP-HPLC of basolateral solutions were hydrolyzed with 6 mol/L hydrochloric acid/1% phenol in evacuated tubes at 110 °C for 24 h. Amino acid analyses were carried out with an analyzer type 4151 Alpha Plus (LKB Biochrom Ltd., Cambridge Science Park, GB) using the ion-exchange resin Ultrapac 8 and sodium citrate buffer system (LKB Biochrom Ltd.). Calibration was achieved by running a mixture of standard amino acids (Merck).

Results and Discussion

Ala-Trp, Val-Phe, and Val-Tyr showed a high in vitro ACE-inhibitory activity having IC$_{50}$ values of 6.5, 51.0 and 12.2 μmol/L, respectively. These results were in the order of magnitude of data reported in the literature. These highly active dipeptide sequences are encrypted in different food proteins which are potential precursors of these ACE-inhibitors: Ala-Trp in bovine α$_{s1}$-casein (Meisel et al., 2006), wakame (Undaria pinnatifida) (Suetsuna and Nakano, 2000), Val-Phe in bovine milk proteins (caseins, α-lactalbumin, β-lactoglobulin) (Maruyama et al., 1987; Philantoleppälä et al., 2000; Schlotthauer et al., 1999), sardine muscle (Matsufuji et al., 1994), wakame (Suetsuna et al., 2004), Val-Tyr in bovine milk proteins (β-casein, β-lactoglobulin) (Kohmura et al., 1990; Abukabar et al., 1998), sardine muscle (Matsui et al., 2002), wheat germ (Matsui et al., 2000), pea albumin (Vermeirssen, 2003), sake (Saito et al., 1994) and wakame (Suetsuna et al., 2004). Inhibition with synthetic dipeptides has previously been reported by Cheung et al. (1980) during studies investigating the binding of peptide substrates and inhibitors to ACE.

Caco-2 cells differentiated and formed monolayers when maintained in culture under standard conditions over a prolonged period of 10 d. Incubation of Caco-2 monolayers for 4 to 24 h with 0.01–1.0 mmol/L Ala-Trp, Val-Phe, and Val-Tyr, respectively, caused no adverse effects with regard to monolayer integrity, tight junction permeability, and activity of the brush-border enzyme DPP IV. All calculated activity indices showed values over 0.7 (Table I), which indicated that Ala-Trp, Val-Phe, and Val-Tyr, respectively, showed no cytotoxic effects on differentiated Caco-2 cells.

During the transport study Caco-2 monolayers in test wells as well as negative control remained intact for 4 h at 37 °C as confirmed by TEER measurements; mean values in the range of 225 to 384 Ω cm$^2$ (SEM$_{max}$: ± 10 Ω cm$^2$) were calculated.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Time</th>
<th>(A) TEER index ± SEM</th>
<th>Activity index$_{DPPIV}$ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.01 mmol/L</td>
<td>0.1 mmol/L</td>
</tr>
<tr>
<td>Ala-Trp</td>
<td>04 h</td>
<td>(A) 1.00 ± 0.006</td>
<td>0.89 ± 0.017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 0.886 ± 0.003</td>
<td>0.928 ± 0.040</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>(A) 0.96 ± 0.014</td>
<td>0.89 ± 0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 0.867 ± 0.027</td>
<td>0.805 ± 0.020</td>
</tr>
<tr>
<td>Val-Phe</td>
<td>04 h</td>
<td>(A) 1.01 ± 0.004</td>
<td>1.01 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 0.918 ± 0.032</td>
<td>0.906 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>(A) 0.98 ± 0.014</td>
<td>1.01 ± 0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 0.799 ± 0.011</td>
<td>0.781 ± 0.040</td>
</tr>
<tr>
<td>Val-Tyr</td>
<td>04 h</td>
<td>(A) 0.93 ± 0.017</td>
<td>0.94 ± 0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 0.970 ± 0.100</td>
<td>0.920 ± 0.129</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>(A) 0.85 ± 0.016</td>
<td>0.82 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) 1.019 ± 0.121</td>
<td>0.971 ± 0.053</td>
</tr>
</tbody>
</table>

Table I. Effects of 4 h and 24 h treatment with 0.01 to 1.0 mmol/L dipeptides on (A) transepithelial electrical resistance and (B) dipeptidyl aminopeptidase IV activity of differentiated Caco-2 cells (monolayers). Results are given as (A) TEER index ± SEM and (B) activity index$_{DPPIV}$ ± SEM; stimulation, > 1; inhibition, < 1; negative control, TEER > 400 Ω cm$^2$; extinction > 1.1.
Fig. 1. RP-HPLC (C_{18}) pattern of samples from the basolateral compartments of Caco-2 monolayers following incubation with 4 mmol/L of the ACE-inhibitory dipeptides Ala-Trp, Val-Phe, and Val-Tyr, respectively, in comparison with negative control (2-morpholinoethanesulfonic acid, MES-HBSS). The elution positions of dipeptides and the corresponding detectable aromatic amino acids are highlighted by arrows.

The differentiated Caco-2 cells (monolayers) transported Ala-Trp, Val-Phe, and Val-Tyr, respectively (Fig. 1). In addition to the intact dipeptides, the corresponding aromatic amino acids have been found in the basolateral samples. Thus, dipeptides were partially hydrolyzed during transport. As expected, peaks of intact dipeptides and corresponding aromatic amino acids were not detectable in negative control samples (Fig. 1). The signal-to-noise ratio of each dipeptide was ≃ 3 indicating that transport of these ACE-inhibitory dipeptides through intact Caco-2 monolayers was clearly detectable. Based on the calculated calibration functions, intact Caco-2 monolayers transported 1.7, 19.5, and 34.9 μg peptides per basolateral compartment, which corresponds to 0.6, 7.5, and 13.4 μg/mL (2.3, 28.4, 47.9 μmol/L) Ala-Trp, Val-Phe, and Val-Tyr, respectively. Therefore, up to 2% of the apically added dipeptides were permeable through Caco-2 monolayers, which is similar to the results obtained in transport studies with other peptides. Comparable results were reported by Satake et al. (2002) who showed that intact Caco-2 monolayers transported about 1 to 2% of the apical added tripeptide Val-Pro-Pro into basolateral compartments. Val-Pro-Pro has been found to be highly resistant to intestinal enzymes and was transported predominantly via the paracellular route (Satake et al., 2002). Transepithelial transport of the hexapeptide Ala-Leu-Pro-Met-His-Ile-Arg, was reported by Vermeirssen et al. (2002), after qualitative analysis by mass spectrometry (MALDI-TOF). Shimizu et al. (1997) investigated the passage of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) across intact Caco-2 monolayers and reported that the efficiency of the transepithelial transport differs between different series of experiments, although this nonapeptide was resistant to hydrolysis by cellular peptidases. In their studies, 400 ng/(h cm^2) bradykinin was detected in samples of basolateral compartments by RP-HPLC after addition of 400 μg/mL bradykinin into the apical compartment. In previous studies (unpublished results), we have found that, in contrast to results of Shimizu et al. (1997), that bradykinin was partly hydrolyzed by brush-border enzymes of differentiated Caco-2 cells, and the predominant degradation product was identified as Gly-Phe-Ser-Pro-Phe-Arg.

The stability of the dipeptides Ala-Trp, Val-Phe, and Val-Tyr, respectively, in human blood serum was determined using RP-HPLC (Fig. 2) as well as
Fig. 2. RP-HPLC (C₁₈) pattern of the ACE-inhibitory dipeptides Val-Phe, Val-Tyr, and Ala-Trp, respectively, after incubation (I) in human blood serum (sulfosalicylic acid-soluble part of ~ 2 mg/mL dipeptide-blood serum mixture) for 0 and 30 min, respectively, in comparison with negative control (human blood serum). The main part of sulfosalicylic acid-soluble serum components elute in the void volume. The elution positions of dipeptides and tryptamine are highlighted by arrows.

by using GP-HPLC (data not shown). No degradation products of Val-Phe or Val-Tyr were detectable after 30 min of incubation with human blood serum. However, Ala-Trp was partly degraded (approx. 10% decrease of peak area) during 30 min of incubation. The main degradation product of Ala-Trp could be identified according to its RP-HPLC elution position as the Trp decarboxylation product tryptamine which was also present as a minor component in human blood serum. In contrast to the serum stability of these dipeptides, in previous studies (Walsh et al., 2004) it was found, that the heptapeptide Ala-Leu-Pro-Met-His-Ile-Arg corresponding to β-lactoglobulin (f 142–148) (Mullally et al., 1997) was rapidly degraded during incubation with human blood serum to several (~ 10) inactive degradation products.
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