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Rapid Communication

Intestinal Transport of the Lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe Monolayer

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Abstract: ACE inhibitory peptides are biologically active peptides that play a role in blood pressure regulation. When derived from food proteins during food processing or gastrointestinal digestion, these peptides could function as efficient agents in treating and preventing hypertension. However, in order to exert an antihypertensive effect by inhibition of the ACE enzyme, they have to reach the bloodstream intact. The aim of this research was to assess if the known ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, derived from a tryptic digest of β-lactoglobulin, could be absorbed through a Caco-2 Bbe cell monolayer in an Ussing chamber and reach the serosal side undegraded. Samples of the mucosal compartment showed high ACE inhibitory activity. No or only little ACE inhibitory activity was detected in the serosal compartment. However, when the serosal sample was concentrated three-fold, a substantial ACE inhibitory activity was registered. Concomitantly, HPLC and MS clearly showed the presence of Ala-Leu-Pro-Met-His-Ile-Arg in the mucosal compartment, whereas in the serosal compartment only MS was able to detect the heptapeptide. In conclusion, under the observed experimental conditions, the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg was transported intact through the Caco-2 Bbe monolayer, but in concentrations too low to exert an ACE inhibitory activity.

Keywords: ACE inhibitory peptide; lactokinin; intestinal transport; hypertension; Caco-2; whey protein; Ussing chamber

Bioactive peptides are food derived peptides that have, besides their nutritional, a functional effect in the human body: they can behave as regulatory components with a hormone like activity [1]. In this respect intestinal transport of active peptides is often a requisite. ACE inhibitory peptides are bioactive peptides with possible blood pressure lowering effects *in vivo*. By inhibiting the angiotensin converting enzyme (ACE), the concentration of the vasoconstrictor angiotensin II decreases, while the concentration of the vasodilator bradykinin increases, which results in an antihypertensive effect [2]. ACE inhibitory peptides have already

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been found in several food protein hydrolysates and ferments [3,4] and have been shown to lower the blood pressure in spontaneously hypertensive rats and hypertensive patients [5,6]. Therefore, there is a growing interest in using these bioactive peptides as efficient agents in treating and preventing hypertension.

ACE inhibitory peptides exert an antihypertensive effect if they reach the bloodstream intact. Therefore, they have to show some resistance to gastrointestinal proteases and brush border peptidases and they have to be absorbed through the intestinal wall with preservation of their physiological activity. The peptide under scrutiny in the present study, Ala-Leu-Pro-Met-His-Ile-Arg, is a lactokinin, a whey protein derived ACE inhibitory peptide, corresponding to a tryptic fragment of bovine β-lactoglobulin, with an IC_{50} value of 42.6 μM. This peptide is resistant to further digestion with pepsin and is only hydrolysed to a very low extent with chymotrypsin [7]. Thus, this peptide may reach the brushborder membrane intact. Passage of intact peptides across the intestinal mucosa may occur via both paracellular and transcellular routes [8]. It is generally accepted that di- and tripeptides are transported, because these are the substrates for the intestinal peptide transporter PEPT1 [9,10]. There is also evidence that larger peptides may be absorbed through the small intestine [11–13]. However, it remains to be established to what extent this occurs and what the transport mechanism is.

In an Ussing chamber experiment we investigated whether the ACE inhibitory heptapeptide could be transported through a Caco-2 cell clone, the Caco-2 Bbe (morphologically homogeneous, brush border expressing cells) cell monolayer, which is a good model for the small intestinal epithelium [14,15]. Intact transport was measured by an *in vitro* ACE inhibition assay and by HPLC and MS.

Addition of 1 mM peptide to the mucosal compartment evoked a small increase in short-circuit current ΔI_{sc} of 0.4 ± 0.2 μA/cm^2. This could indicate the transport of the positively charged heptapeptide (Arg), transport of its charged degradation products or ion transport induced by the heptapeptide. The transepithelial electrical resistance was about 300 Ω.cm^2 and remained constant during the whole experiment, which reflects the structural integrity of the cell monolayer [15]. Addition of 10 mM L-glutamine to the mucosal bathing solution at the end of the experiment induced an increase in short-circuit current ΔI_{sc} of 1.4 ± 0.9 μA/cm^2, as a result of the cotransport with sodium. This observation of active transport guarantees the viability and functionality of the cell monolayer [15].

In the Ussing chamber without added peptide, neither ACE activity nor ACE inhibitory activity was observed in the mucosal or serosal compartment. Hence, the Caco-2 Bbe cell monolayers did not secrete ACE enzyme or ACE inhibitory compounds. The mucosal bathing solutions of all six Ussing chambers in which the ACE inhibitory peptide was supplemented, showed high ACE inhibitory activity (Figure 1). This could be due to the heptapeptide or to active degradation products. Brushborder peptidases like aminopeptidase N and dipeptidylpeptidase IV, which are expressed in Caco-2 cells, are able to cleave the heptapeptide. Hereby Leu-Pro can be formed which has ACE inhibitory activity as well [16]. Little to no ACE inhibitory activity was detected in the serosal bathing solutions. However, when the samples were concentrated three-fold, the ACE inhibitory activity in the serosal bathing solutions increased to 30%. Hereby, the effect of the three-fold concentrated modified Krebs buffer on the ACE inhibitory activity was taken into account.

HPLC analysis of the mucosal samples yielded three significant peaks: peaks 1–2 with a retention time of around 4 min and a total area of 28% and peak 3 with a retention time of 29 min and an area of 59%. In the serosal samples only peaks 1–2 were detected after 4 min elution with a total area of 99%. Peaks 1–2 correspond to hydrophilic compounds present in the buffer solution like glucose and mannitol, while the last peak presents the heptapeptide. This peak closely resembled the one in the control HPLC chromatogram of the

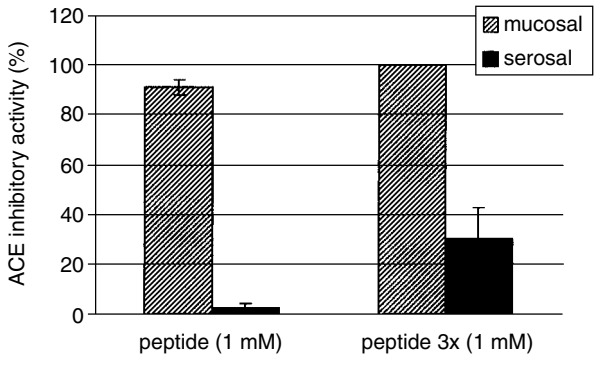

Figure 1 ACE inhibitory activity (%) observed in the mucosal and serosal compartment of the Ussing chambers with Caco-2 Bbe cell monolayer upon administration of the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (1 mM), analysed as such ($n = 6$) and three times concentrated ($n = 2$) in the ACE inhibition assay.

heptapeptide after synthesis (data not shown). Thus, HPLC analysis clearly detected the pure peptide in the mucosal compartments, but not in the serosal compartments. However, the highly sensitive MALDI-TOF mass spectrometry demonstrated the presence of the heptapeptide in both the mucosal and serosal samples. The mass spectra of both the mucosal and serosal samples revealed the presence of a peak at mass 837, which is the molecular mass of the heptapeptide (836) plus a proton (Figure 2). The mucosal sample contained a few other high molecular mass peaks: 859 is the mass of the heptapeptide plus sodium, while 881 represents the heptapeptide plus disodium. The peak with mass 853 in the serosal sample represents the peptide plus proton in which methionine has been oxidized.

Several *in vivo* studies have demonstrated the antihypertensive effect of orally administered food derived ACE inhibitory peptides [5,6]. *In vitro* experiments may provide an outcome as laboratory-scaled alternatives for experimentation with humans or animals in the screening process for ACE inhibitory peptides. In this respect, the intestinal transport of ACE inhibitory peptides is an important facet in the *in vitro* investigation of the maintenance of ACE inhibitory activity through the oral pathway. Only one study describes the *in vitro* transepithelial transport of bioactive oligopeptides [17]. It concludes that the susceptibility to the brushborder peptidases controls the transport rate and that both paracellular transport and transcytosis may participate in oligopeptide passage across the intestinal epithelium.

In this *in vitro* study, the mass spectrometry results combined with the electrophysiological and ACE inhibition data, show that transport of Ala-Leu-Pro-Met-His-Ile-Arg through the Caco-2 Bbe monolayer took place within 10 min of administration. Although it cannot be excluded that some degradation of the heptapeptide occurred or that transport of degradation products took place, these results demonstrate that the ACE inhibitory heptapeptide is transported intact, albeit in very low concentrations. As samples were taken 10 min after addition of the ACE inhibitory peptide to the mucosal compartment, there may have been insufficient time for the transport to occur completely. However, longer sampling times could increase the risk of degradation.

Further experiments are required to study the transport over an extended period of time, with different concentrations of peptide, in order to determine the transport kinetics.

EXPERIMENTAL

Peptide

The ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (836 g/mol) was synthesized by solid phase peptide chemistry and Fmoc protection and evaluated by HPLC-MS (purity of 84.37% (w/w)) at the Biotechnology Center of the University of Illinois (Urbana, IL).

Cell Culture

Caco-2 Bbe [14] cells were routinely grown in T-75 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies), containing 10% (v/v) fetal bovine serum (Fischer), 0.25 mg/l fungizone (Life Technologies) and 0.5% (v/v) penicillin-streptomycin (Life Technologies), supplemented with non-essential amino acids (Life Technologies) and 10 mg/l transferrin (Sigma, T 5391), in an atmosphere of 20% O_2 and 5% CO_2 at 37 °C.

For the Ussing chamber experiments 10^5 cells/well (passage 95) were inoculated in Costar Brand Snapwell culture plates (Costar) and maintained for 23 days with daily medium refreshment.

Electrophysiological Studies

The Snapwell inserts with the Caco-2 Bbe monolayer were mounted in modified Ussing chambers (Physiological Instruments Inc.) with an exposed area of 1.13 cm^2 [18]. Cells were bathed on each side with 4 ml oxygenated (95% O_2/5% CO_2) modified Krebs buffer (140 mM Na, 119.8 mM Cl, 25 mM HCO_3, 1.2 mM Mg, 1.2 mM Ca, 4.8 mM K, 2.4 mM HPO_4, 0.4 mM H_2PO_4, pH 7.4) maintained at 37 °C and short circuited after appropriate correction for fluid and system resistance, using dual channel voltage/current clamps (VCC MC2, Physiological Instruments) connected with a computer interface (Acquire & Analyze Software, Physiological Instruments). Serosal solutions were supplemented with 10 mM glucose while mucosal solutions were supplemented with 10 mM mannitol for osmotic balance. Transepithelial potential difference (P_D) and the current required for nullifying the spontaneous transepithelial potential difference (I_c) was monitored. At $P_D = 0$ mV, respective short-circuit currents (I_{sc}) were calculated. Periodic measurements of potential difference and current deflections induced by bipolar pulses of 1 s duration and 66 μA amplitude, served to calculate the transepithelial electrical

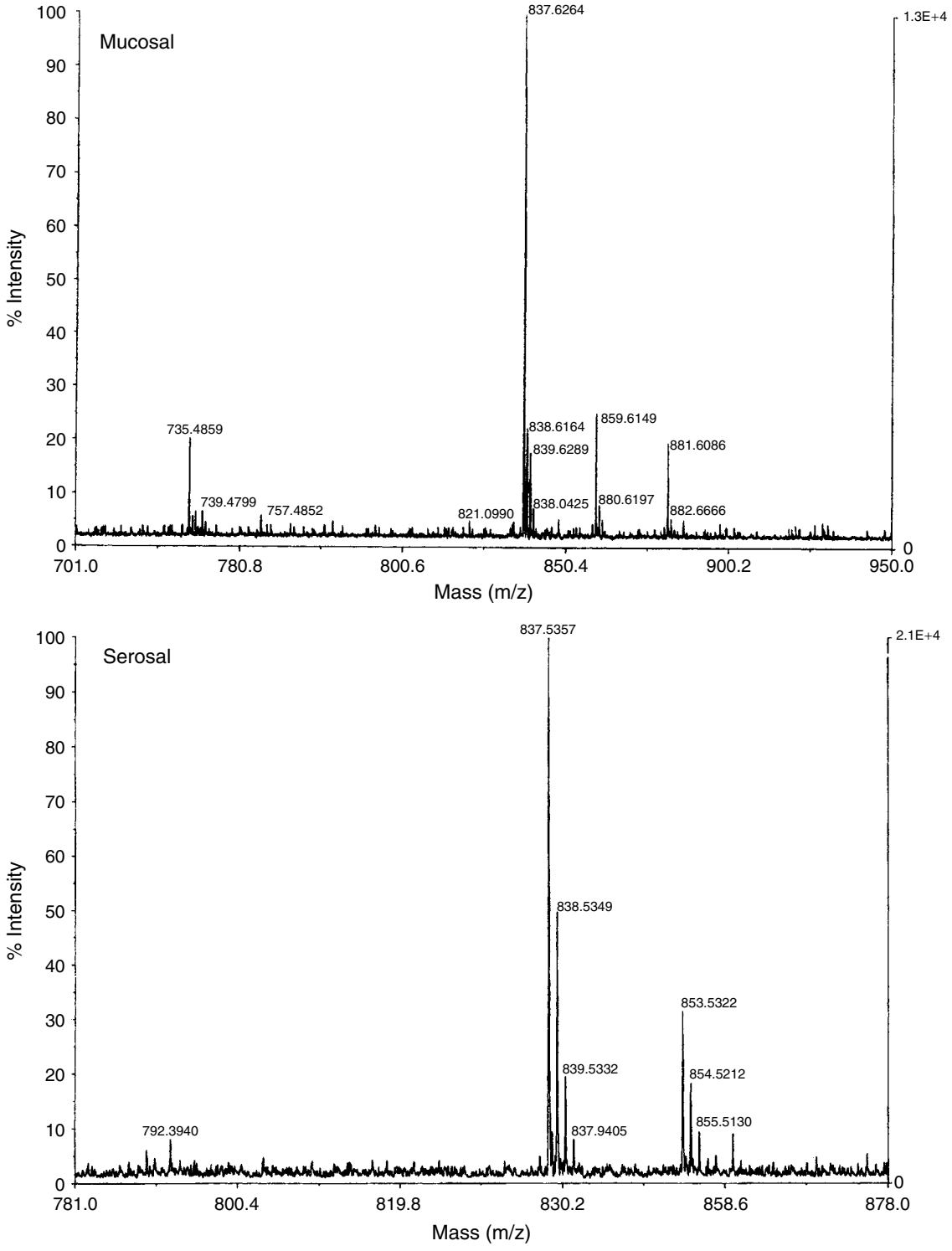

Figure 2 MALDI-TOF mass spectrometry profile of the mucosal and serosal compartment of the Ussing chamber upon administration of the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (1 mM).

resistance (R) based on Ohmic relationship. Ala-Leu-Pro-Met-His-Ile-Arg was added to the mucosal bathing solution in a final concentration of 1 mM while equimolar amounts of mannitol were added to the serosal side ($n = 6$). One Ussing chamber was left without added peptide and used as a control. After 10 min, samples were taken from both the mucosal and serosal side for the ACE inhibition assay and HPLC-MS. Samples were freeze-dried and redissolved prior to analysis. In the Ussing chambers, samples were replaced by equal volumes of buffer solution and 10 mM L-glutamine was added to the mucosal compartments together with 10 mM mannitol to the serosal compartments, to check for an I_{sc} increase indicating cell monolayer viability and integrity during the incubation period. Responses to substrate supplementation were expressed as differences between basal value and maximal response (ΔI_{sc}).

ACE Inhibitory Activity and ACE Activity

Lyophilized samples were dissolved to the original volume in demineralized water, except for the concentrated analysis in two Ussing chambers, where the lyophilized powder was dissolved in a volume three-fold smaller than the original one. The ACE inhibitory activity was measured by an ACE inhibition assay based on the spectrophotometric method described by Holmquist *et al.* [19]. The reaction mixture contained 500 µl ACE reagent (Sigma, 305-10), reconstituted with only 5 ml demineralized water, 300 µl demineralized water (blank) or inhibitor solution (sample) and 300 µl ten times diluted rabbit lung acetone powder (Sigma, L 0756) extract as ACE source [20]. After 5 min incubation at 37 °C, the decrease in absorbance over another 5 min incubation at 37 °C was measured against demineralized water at 340 nm with an UV/VIS double beam spectrophotometer (Uvikon 922, Kontron Instruments). The decrease in absorbance (y) corresponded to an ACE activity (x) as determined by the standard curve $y = 0.0031x + 0.011$, which was constructed using different dilutions of the ACE Control-E (Elevated) (Sigma, A 7040) as enzyme source. ACE activity and ACE inhibitory activity were expressed as a percentage, assuming that the ACE activity and inhibitory activity of the blank were respectively 100% and 0%. To assess the ACE activity in the control, which accounted for the enzyme released by the cell monolayer in the buffer solution, 300 µl of mucosal and serosal bathing solution respectively, was used as the enzyme source in the ACE inhibition assay. Demineralized water was added instead of an inhibitory compound.

HPLC and MS

The bathing solutions of two Ussing chambers in which the heptapeptide was supplemented, were analysed by HPLC and MS.

Reversed-phase HPLC analysis was performed on a Beckman C_{18} Ultrasphere column (150 × 4.6 mm, 5 µm) and a Beckman HPLC equipment model 126 with a UV detector operating at 215 nm. A 40 µl aliquot of 1 mg/ml lyophilized powder in 0.1% (v/v) TFA in water was loaded onto the column and eluted at a flow rate of 0.5 ml/min with a linear gradient from 100% solvent A (0.06% TFA in water) to 80% solvent B (0.052% TFA in acetonitrile) in 60 min at 25 °C.

Samples were also analysed by Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) spectrometry on a Voyager-DE STR (Applied Biosystems) using positive reflection mode with 100 ns delayed extraction, an accelerating potential of 20 kV (76% grid voltage, 0% guide wire voltage) and external calibration to convert the time-of-flight to mass. Prior to analysis, the samples were acidified with aqueous TFA to pH <4 and a final TFA concentration between 0.1% and 1%, subsequently concentrated and desalted using C_{18} Zip Tip (Millipore) and finally crystallized with α-hydroxycinnamic acid (Fluka).

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