Research Article

Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides. PepT1-mediated flux of Tyr-Pro-Ile

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This paper examines the *in vitro* transepithelial transport of antihypertensive peptides derived from egg proteins using Caco-2 cell monolayers. Ovokinin (FRADHPFL) was absorbed intact through the Caco-2 cell epithelium, although it was also susceptible to the action of brush-border aminopeptidases that yielded shorter fragments prior to their transport. The tripeptide YPI was resistant to cellular peptidases and transported through the monolayer, what suggests that the reduction in systemic blood pressure caused by this peptide may be mediated by effects at tissue level. Its pathway for transepithelial absorption was examined using inhibitors of the different mechanisms for oligopeptide transport in the intestinal tract. The main route involved in the transepithelial flux of YPI is probably the peptide H^+-coupled transporter PepT1. These results highlight the potential of antihypertensive peptides to be used in the formulation of functional foods.

Keywords: Antihypertensive peptides / Caco-2 cells / Egg proteins / Intestinal transport

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1 Introduction

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions and conditions that may ultimately affect health. Numerous peptides exhibiting opiate, immunomodulatory, antimicrobial, antioxidant, antithrombotic and antihypertensive activities have been reported [1]. The physiological effects of bioactive peptides depend on their ability to reach their target sites in an intact form. In this context, gastrointestinal digestion plays a key role in the formation and degradation of bioactive peptides [2–4]. Many of the known bioactive peptides are not transported from the gastrointestinal tract into the blood stream. These peptides may operate by modulating nutrient absorption and influencing the gastrointestinal function, either through a direct effect in the gut lumen (*e.g.* caseinphosphopeptides), or through binding to receptors on the intestinal cell wall (*e.g.* opiod peptides) [1]. For other peptides, such as the antihypertensive peptides that inhibit angiotensin I-converting enzyme (ACE), absorption through the intestinal epithelium to get to the peripheral organs is a requirement [5]. In this case, their molecular mass, hydrophilicity and tendency to undergo aggregation or degradation by intestinal brush-border peptidases greatly determine their bioavailability and physiological effect [2, 6].

There are several mechanisms for transepithelial oligopeptide transport in the intestinal tract. The absorption of small oligopeptides arising from digestion of dietary proteins can be mediated by PepT1, a proton-coupled membrane transporter [7]. PepT1 has been described to carry, not only di- and tri-peptides, but also peptide-like β-lactam antibiotics, orally active ACE inhibitors and a variety of peptidomimetic drugs [7–9]. Other absorption routes such as paracellular transport through the intercellular junctions have been described. This is the case, for instance, of the antihypertensive tripeptide VPP [10]. Masuda *et al*. [11] detected VPP in the abdominal aorta of spontaneously

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Abbreviations: ACE, angiotensin-converting enzyme; **AP,** apical; **BL,** basolateral; **HBSS,** Hank's balanced salt solution; **SHR,** spontaneously hypertensive rats; **TEER,** transepithelial electrical resistance

hypertensive rats (SHR), which strongly supports that it is transepithelially transported. Finally, transcytosis *via* internalized vesicles is regarded as the main transport system for long-chain oligopeptides [12, 13]. Absorptive transcytosis has been suggested to be the major transport mechanism of the well-known physiologically active peptide bradykinin [14].

In previous studies, we identified, in a peptic hydrolysate of egg white, peptides, such as FRADHPFL (previously named ovokinin by Fujita *et al*. [15]), RADHPFL and YAEERYPIL, which inhibit ACE *in vitro* and exhibit antihypertensive activity in SHR at a minimum effective dose of 2 – 4 mg/kg [16, 17]. The bioactive peptides RADHPFL and YAEERYPIL are cleaved, under simulated gastrointestinal digestion conditions, giving the shorter fragments RADHP, YAEER and YPI, which do not exhibit ACE-inhibitory activity, but maintain their blood pressure lowering effect [18]. Further work showed that RADHPFL, YAEERYPIL, as well as the end products of their gastrointestinal digestion, RADHP and YPI, induce a strong relaxation in endothelium-denuded rat aortic rings [19]. The objective of this study was to investigate whether these peptides are resistant to brush-border peptidases and susceptible to intestinal transepithelial transport. For this purpose we used Caco-2 cell monolayers, that express many intestinal enzymes and exhibit different transport mechanisms and have been broadly used as models for the small intestine epithelium [14].

2 Materials and methods

2.1 Materials

The synthetic peptides FRADHPFL, RADHPFL, RADHPF, RADHP, YAEERYPIL, YAEER and YPI were obtained by conventional Fmoc solid phase synthesis with a 431 peptide synthesizer (Applied Biosystem, Überlingen, Germany) and their purity was verified by RP-HPLC-MS/MS. Captopril, cytochalasin D, wortmannin, glysil-sarcocine (Gly-Sar) and bradykinin were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2 Cell cultures

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM, supplemented with 10% fetal bovine serum, 1% nonessential amino acids and antibiotics, at 37°C in a humidified atmosphere containing 5% CO_2. All the cells used in this study were between passages 30 and 45. For the experiments, cells were seeded onto a six-well Transwell clear polyester permeable membrane support (0.4 µm pore size, 24 mm diameter, 4.7 cm^2 grown surface, Costar, Corning, NY, USA), at a density of 10^5 cell/cm^2. The culture medium was replaced every other day and allowed to differentiate for at least 21 days before the experiments. Caco-2 monolayers with an integrity equivalent to a transepithelial electrical resistance (TEER) higher than 200 Ω/cm^2 were used for the transport studies.

2.3 Transport studies

All solutions were preheated before the experiments. Cells growing in DMEM were gently rinsed with Hank's balanced salt solution (HBSS) and equilibrated for 30 min at 37°C prior to transport experiments. Transepithelial transport of pure synthetic peptides was evaluated adding peptides (1 mM) dissolved in HBSS either to the apical (AP) or basolateral (BL) side. The chamber was incubated at 37°C for different periods and the AP and BL solutions were taken for RP-HPLC-MS/MS analyses. For inhibition experiments, Gly-Sar (10 mM) was dissolved in HBSS, and wortmannin (500 nM) and cytochalasin D (0.5 µg/mL) were dissolved in DMSO (final concentration in the medium 0.044%). The cell monolayers were incubated for 30 min with the inhibitors or with DMSO, as a control, before the peptide transport experiments.

2.4 Analysis by online RP-HPLC-MS/MS

The AP and BL solutions were analysed on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected online to an Esquire-LC quadrupole IT instrument (Bruker Daltonik, Bremen, Germany). The HPLC was equipped with a quaternary gradient pumping system, an in-line degasser, a variable wavelength absorbance detector set at 220 nm, and an automatic injector (all 1100 Series, Agilent Technologies). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (BioRad, Richmond, CA, USA). The injection volume was 50 µL. Solvent A was 0.37 mL/L TFA in milli-Q water and solvent B 0.27 mL/L TFA in ACN. Peptides were eluted with a linear gradient of solvent B in A, from 2 to 10% in 15 min, 10 to 20% in 35 min and 20 to 30% in 20 min. The flow rate was 0.8 mL/min. The flow was split postdetector by placing a T-piece (Valco, Houston, TX, USA) connected with a 75 µm id peek outlet tube of an adjusted length to give, approximately, 20 µL/min of flow directed into the mass spectrometer *via* the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (m/z) range 100 – 1500. About 25 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto-MS(n) analyses was 5000 and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis™ (version 3.0; Bruker Daltoniks), the m/z spectral data were processed and transformed to spectra representing

mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing.

2.5 Statistical analysis

All values are presented as means ± SEM. Means were compared using Student's *t*-test. Differences with *p*-values <0.05 were considered significant. Analysis was performed using the Graph Pad Prism 4 software.

3 Results and discussion

3.1 Resistance of the antihypertensive peptides to epithelial peptidases

The resistance to the enzymatic activity of epithelial peptidases of antihypertensive peptides derived from egg white was examined using the Caco-2 monolayer model. For this purpose, the synthetic peptides were added to the AP chamber, and after a 60 min incubation period at 37°C, the AP and BL contents were analysed by RP-HPLC-MS/MS. The integrity of the monolayers was checked before and after the experiments. TEER values reached 420 Ω/cm^2 and no reduction was observed by effect of the incubation with the peptide. Table 1 summarizes the results obtained. Bradykinin (RPPGFSPFR) was used as a control in our system. This peptide was transported intact, in agreement with previous reports that demonstrated that it is not hydrolysed by brush-border peptidases and crosses the transepithelial barrier of Caco-2 cells [14, 20]. Bradykinin is thought to be adsorbed to the AP cell membrane through hydrophobic interactions, internalized by the cells and transported across the layer, following a transcytotic route [14].

As illustrated in Fig. 1, when FRADHPFL (ovokinin) was incubated in the AP chamber, the RP-HPLC-MS/MS analysis showed that, in addition to the intact octapeptide, there were smaller peptides that resulted from a sequential hydrolysis at the N-terminus, presumably by brush-border peptidases. Caco-2 cells express brush-border aminopeptidases, including dipeptidyl aminopeptidases, which might be able to cleave certain N-terminal residues [14]. All these peptides, except for RADHPFL, were transported to the BL chamber (Table 1). The observation that intact FRADHPFL

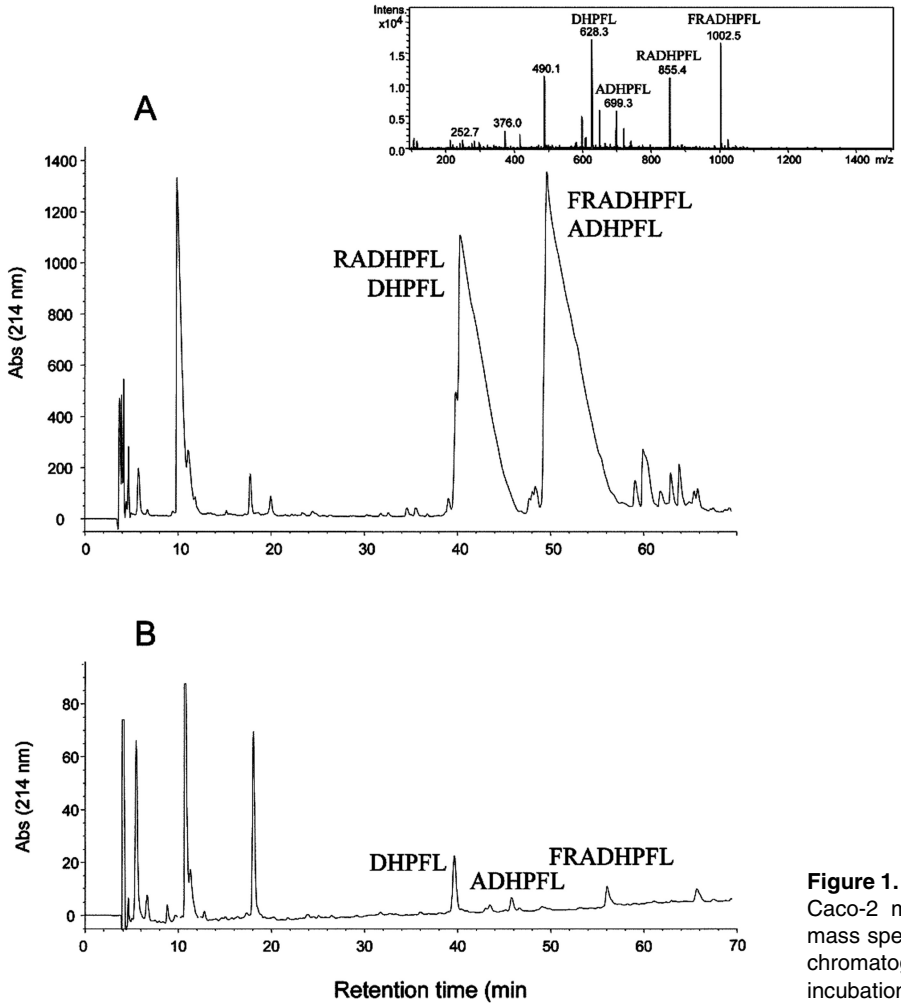

Figure 1. Transport of FRADHPFL through the Caco-2 monolayer. (A) UV-chromatogram and mass spectrum of the AP chamber, and (B) UV-chromatogram of the BL chamber, after 60 min of incubation.

Table 1. Peptide fragments found by RP-HPLC-MS/MS in the AP and BL chambers during transport studies of bradykinin (RPPGFSPFR) and antihypertensive egg-derived peptides through Caco-2 monolayers

Peptide	Peptides found in the AP chamber	Peptides found in the BL chamber
RPPGFSPFR	RPPGFSPFR	RPPGFSPFR
FRADHPFL	FRADHPFL, RADHPFL, ADHPFL, DHPFL	FRADHPFL, ADHPFL, DHPFL
RADHP	RADHP	none
YAEERYPIL	YAEERYPIL, EERYPIL	YAE, YPI
YAEER	YAEER	YAEER
YPI	YPI	YPI

At least, three independent experiments were conducted, assaying three wells *per* peptide in each experiment.

was absorbed through a Caco-2 cell monolayer is in accordance with previous data [14].

We next assayed the pentapeptide RADHP, which is the final product of the *in vitro* simulated gastrointestinal digestion of FRADHPFL and exhibits antihypertensive effects *in vivo* in the SHR model [18]. RADHP remained intact in the AP chamber, but analysis of the BL solution showed that it was not absorbed through the Caco-2 monolayer. The lack of RADHP transport in our experimental conditions may suggest that RADHP does not require absorption to exert its antihypertensive action. Yamada *et al*. [21] reported that RADHPF (ovokinin 2-7) and several synthetic analogues exhibited antihypertensive activity at lower doses after oral than intravenous administration to SHR. This suggested that they could lower blood pressure through the interaction with receptors expressed in the gastrointestinal tract, implying that no absorption was required. In contrast to these results, Scruggs *et al*. [22] found that intravenous administration of RADHPF lowered blood pressure in Sprague-Dawley rats at a low threshold dose of 0.1 mg/kg. In any case, it should be mentioned that FRADHPFL, RADHPF and RADHP were reported to lower blood pressure through different modes of vasorelaxing activity mediated by bradykinin B_1 or B_2 receptors [19, 22 – 24] and that these receptors are present in various types of cells including the colonic epithelial cells [25].

When YAEERYPIL was incubated in the AP chamber it was partially hydrolysed by brush-border peptidases that released the fragment EERYPIL. The RP-HPLC-MS/MS analysis of the BL solution suggested that YAEERYPIL and EERYPIL were further hydrolysed, presumably by intracellular peptidases, because only the shorter fragments YAE and YPI were transcellularly transported (Table 1). On the other hand, the peptides arising from the *in vitro* simulated digestion of YAEERYPIL: YAEER and YPI [18], were also tested in the same manner. Both peptides crossed the monolayer and no derived peptides were found in the AP or BL chamber (Table 1). Previous studies revealed that, unlike YAEER, YPI had a significant antihypertensive effect in SHR [18]. The present results show that YPI exhibited resistance to epithelial cell peptidases and that it was transepithelially transported across the Caco-2 monolayer. This makes YPI a potential candidate to be absorbed by the intestinal epithelium and reach the bloodstream and thus, we further evaluated the mechanism involved in its absorption.

3.2 Transport of YPI

The tripeptide YPI was selected to further study its transport mechanism. When incubated in the AP chamber, YPI rapidly reached the BL chamber and its concentration increased up to 60 min (data not shown). Therefore, an incubation time of 60 min was used in the subsequent experiments. Figure 2 shows the amount of YPI transepithelially transported which lays within the same range than that of other peptides described in the literature. For instance, according to Satake *et al*. [10], less than 2% of the ACE-inhibitor and antihypertensive peptide VPP, added to the AP chamber, was transported to the BL chamber in 60 min.

We first evaluated whether passive permeation was the mean absorption route for YPI by comparing the AP-to-BL and the BL-to-AP transport. As shown in Fig. 2, the amount of peptide transported from the BL to the AP chamber was slightly, but significantly, higher than that transported form the AP to the BL chamber. The transepithelial flux of peptides following the passive permeation paracellular route does not normally exhibit differences between both directions [14, 26]. However, differences in the active transport mediated by the AP H^+-coupled peptide transporter and the BL peptide transporter have been previously described [27, 28]. Some orally active ACE inhibitors used in the clinical treatment of systemic hypertension, such as captopril, are substrates for active transport [29, 30]. Therefore, the behaviour of captopril was studied for comparison. Although the percentage of captopril absorbed was higher than that of YPI, its BL-to-AP transport was also higher than the AP-to-BL one (Fig. 2).

In order to discard passive permeation as the main transport route, the transepithelial transport of YPI was measured in the presence of cytochalasin D, which opens tight cell junctions by altering the cytoskeletal structure [31]. The transepithelial flux of compounds that undergo paracellular passive permeation is normally increased in the presence of cytochalasin D [14, 26]. In fact, cytochalasin D treatment reduced TEER values in, approximately, 30%, which indicates that the paracellular route was expanded. As shown in Fig. 3, cytochalasin D did not modify the AP-

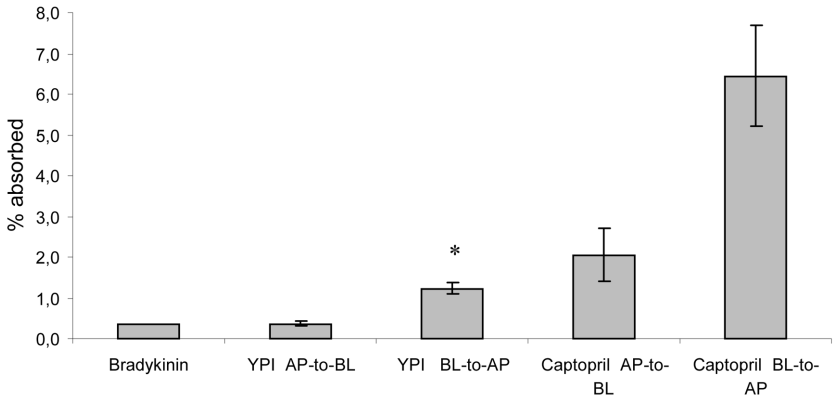

Figure 2. AP-to-BL and BL-to-AP transport of YPI (1 mM) across the Caco-2 monolayer. Results are expressed as the amount of peptide absorbed as percentage of the total peptide added to the AP or BL chamber. Values are means ± SEM of, at least, three independent experiments (three wells *per* peptide in each experiment). *$p < 0.05$ *versus* YPI AP-to-BL; {$p < 0.05$ *versus* captopril AP-to-BL.

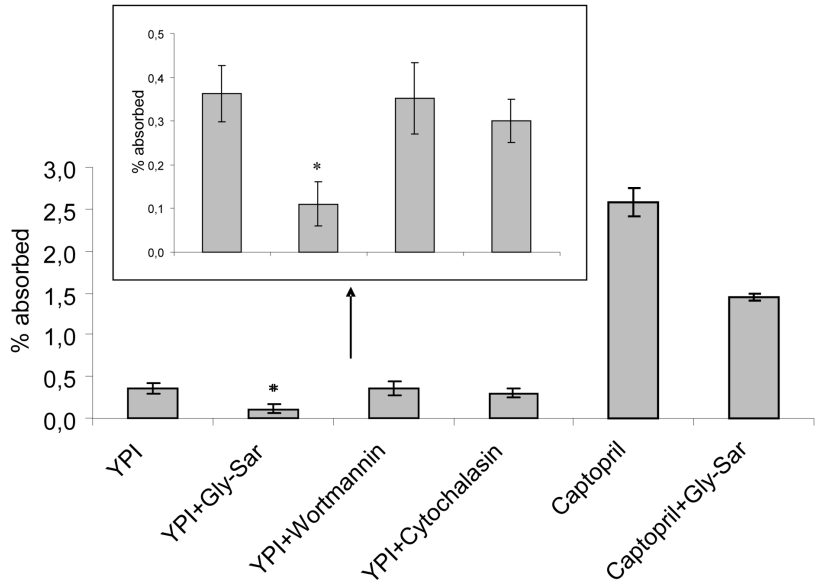

Figure 3. Effect of Gly-Sar, cytochalasin D and wortmannin on the AP-to-BL transport of YPI across the Caco-2 monolayer. Results are expressed as the amount of peptide absorbed as percentage of the total peptide added to the AP chamber. Values are means ± SEM of, at least, three independent experiments (three wells *per* peptide in each experiment). Means were compared using Student's *t*-test. *$p < 0.05$ *versus* YPI; {$p < 0.05$ *versus* captopril.

to-BL transport of YPI, confirming that the paracellular route was not its main mechanism for absorption.

We next addressed whether the absorption of YPI across the cell monolayer was mediated by other of the major mechanisms recognized for the intestinal transport of oligopeptides, the adsorptive transcytosis. The absorption of YPI in the presence of wortmannin, which inhibits the transcytosis in polarized cells [32] was not reduced (Fig. 3), suggesting that the vesicle-mediated transport process was not implicated. Vesicular trafficking has been described as a mechanism of absorption of certain peptides through the epithelial enterocyte [14].

Finally, the tripeptide YPI was incubated in the presence of a ten-fold higher concentration of Gly-Sar, a biologically stable dipeptide, which is a substrate for the H^+-coupled transporter PepT1 [28, 33 – 35]. The absorption of YPI was significantly reduced in the presence of Gly-Sar, suggesting that that the PepT1-mediated transport was involved in the transepithelial transport of YPI. Similarly, the AP-to-BL transport of captopril, a known substrate for the H^+ peptide transporter PepT1 [29, 30] was reduced when it was incubated in the presence of Gly-Sar (Fig. 3).

PepT1-mediated transport is mainly regarded as a degradative pathway for di- and tri-peptides [36, 37]. In fact, it has been reported that the antihypertensive peptide VPP, apically taken *via* PepT1, is quickly hydrolysed by intracellular peptidases producing free Val and Pro [10]. Similarly, the tripeptide Gly-Pro-Hyp is transported *via* the H^+-coupled PepT1 across the porcine intestinal brush-border membrane into Pro-Hyp [37]. While the present results show that YPI was resistant to brush-border peptidases and demonstrate the passage of the intact tripeptide through the Caco-2 cell monolayer, we cannot rule out that is partially hydrolysed by intracellular enzymes to free amino acids. In any case, it should be noted that the activity of PepT1 in Caco-2 cells may be low in comparison with that of epithelial cells isolated from animal intestine, what suggests that the absorption rate for an intact tripeptide *in vivo* would be much higher than the value obtained when used Caco-2 cells as models [10, 38, 39].

4 Concluding remarks

The concept that small peptides can escape total digestion to amino acids, resist brush-border peptidases and be transported from the intestinal lumen into the blood circulation in intact form is rather new, but is gaining acceptance [35]. So far, few studies have described the *in vitro* transepithelial transport of bioactive oligopeptides [5, 10, 14, 20, 26, 40]. Our experimental data confirm that the antihypertensive peptide ovokinin (FRADHPFL) is absorbed intact through a Caco-2 cell epithelium, although it was also susceptible to the action of brush-border aminopeptidases that yielded shorter fragments prior to their transport. The pentapetide RADHP, that is produced from FRADHPFL by a simulated gastrointestinal digestion and exhibits antihypertensive effects *in vitro*, resisted the action of epithelial peptidases, but no evidence could be provided for its absorption. On the other hand, the antihypertensive tripeptide YPI was resistant to cellular peptidases and transported through the monolayer, what suggests that reduction of systemic blood pressure caused by this peptide may be mediated by effects at tissue level. The main route involved in the transepithelial flux of YPI was probably the peptide H^+-coupled transporter PepT1. These results highlight the potential use of antihypertensive peptides in the formulation of functional foods. However, the exact mechanism underlying this effect, and whether YPI can eventually reach the bloodstream to exert systemic effects at tissue level has yet to be elucidated.

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The authors have declared no conflict of interest.

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