Supporting Information

Fuhrmann and Leroux 10.1073/pnas.1100285108

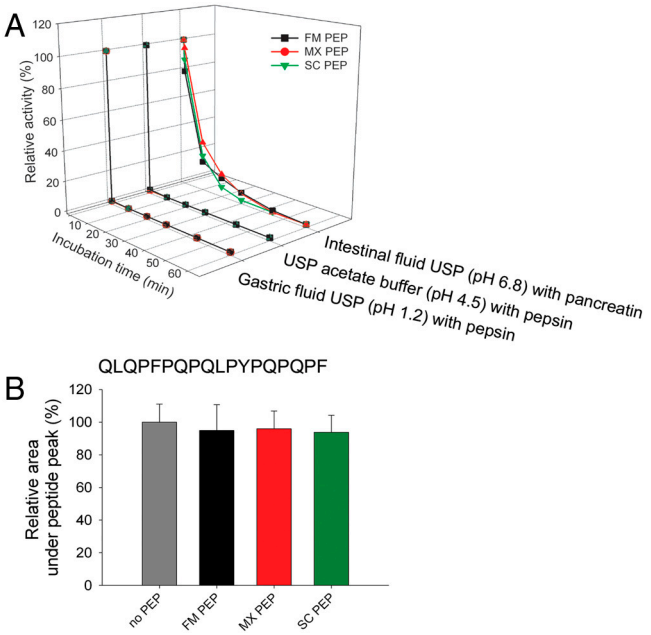


Fig. S1. In vitro stability and activity of individual PEPs. (*A*) Residual enzymatic activity of PEPs after incubation in simulated gastric fluids, pH 1.2 or 4.5 (with pepsin), and in simulated intestinal fluid, pH 6.8 (with pancreatin). The activity was assessed by measuring *Z*-Gly-Pro-*p*NA hydrolysis at 410 nm and was normalized to the activity at time zero. The results are plotted as mean, $n = 3$, and standard deviation (SD) was omitted for clarity (coefficient of variation <25%). (*B*) Gluten proteolysis by PEPs in vitro. PEPs were incubated with whole wheat gluten (1:100, w/w), simulating gastric (pH 4.5 and pepsin for 1 h) followed by intestinal (pH 6.8 with trypsin and chymotrypsin for 2 h) digestion conditions. The relative abundance of the gluten peptide QLQPFPQPQLPYPQPQPF was quantified by LC-MS. Each value was normalized to a non-PEP-treated sample. Mean + SD, $n = 3$.

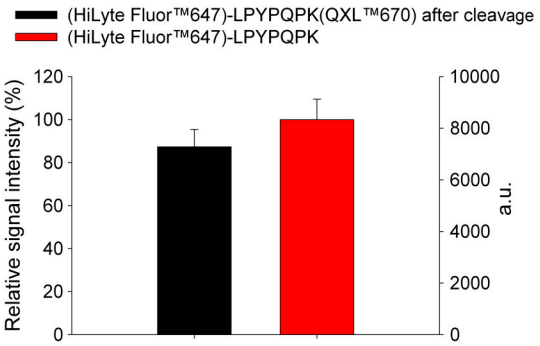


Fig. S2. In vitro fluorescence intensities of (HiLyte Fluor™647)-LPYPQPK(QXL™670) after cleavage and (HiLyte Fluor™647)-LPYPQPK. Both peptides (2.5 μM) were incubated in phosphate buffer (10 mM) with addition of FM PEP (0.005 μg/mL). Maximum fluorescence intensities were measured using a microplate reader and normalized to the maximum signal intensity (a.u.).

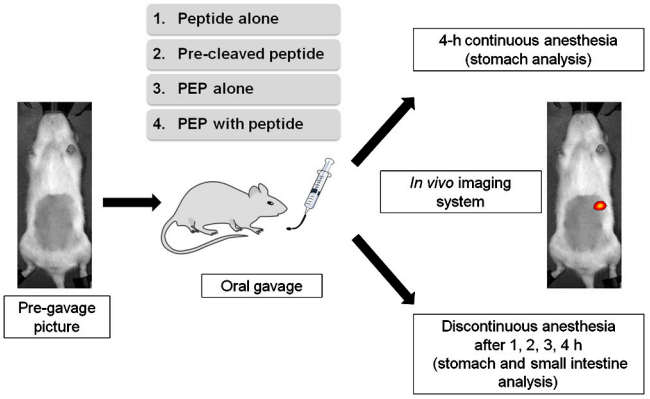


Fig. S3. Schematic illustration of the study protocol. Rats were first shaved and a pregavage picture was taken. Animals were then orally given the labeled peptide alone (negative control 1), the precleaved peptide (positive control), PEP alone (negative control 2), or PEP followed by the labeled peptide (5 min later) (study group). The rats were anesthetized continuously for 4 h (prolonged exposure to stomach conditions) or discontinuously 1, 2, 3, and 4 h after oral gavage (analysis of the stomach and small intestine). In the latter scenario, rats were anesthetized for 15 min at each time point and awake between measurements.

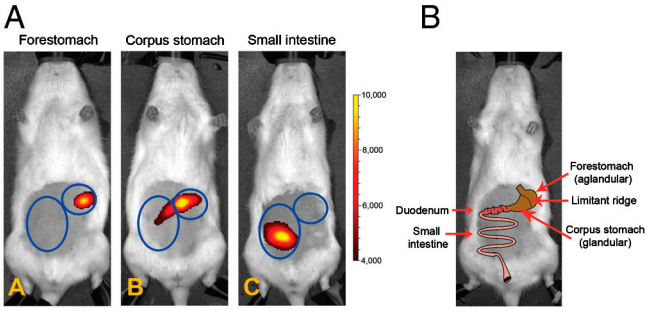


Fig. S4. Illustration of how images were interpreted and analyzed. (*A*) Regions of interest (ROIs) were placed over areas representing the stomach (small round ROI) and the small intestine (large oval ROI). ROIs were kept constant throughout sets of experiments in terms of size, form, and position on the rat's abdominal area. In *A*, the cleaved peptide is located in the forestomach. *B* shows the signal in the main (corpus) stomach while in *C* the peptide moved into the small intestine. Color bars are identical. (*B*) Simplified schematic anatomy of the rat GI tract, indicating fore stomach and corpus stomach separated by the limitant ridge, a low tissue fold.

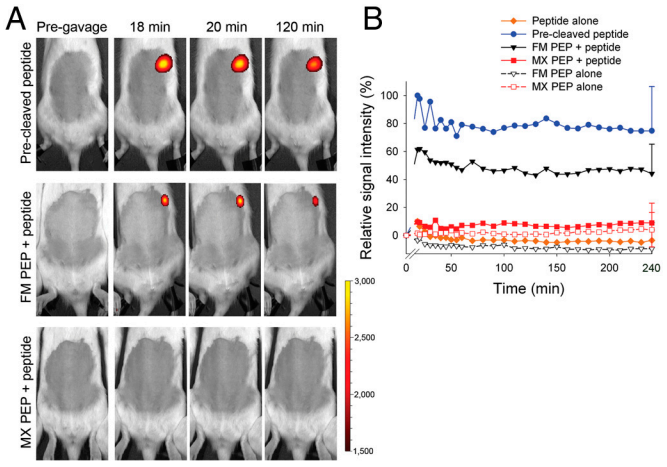


Fig. S5. Evolution of fluorescence intensity during imaging in the stomach under continuous anesthesia. Rats were orally administered the peptide alone, the precleaved peptide, and FM and MX PEP with or without peptide. (*A*) Relative signal intensity after the oral administration of precleaved peptide and PEPs with peptide. Pictures were taken before and 18, 20, and 120 min after oral gavage. The pictures shown are from representative rats in each set. Color scales are identical for all pictures. (*B*) Signals are plotted by setting the average in vivo maximum signal (precleaved peptide at $t = 18$ min) to 100%. Mean, $n = 6$; for clarity, SDs are shown for the last time point only.

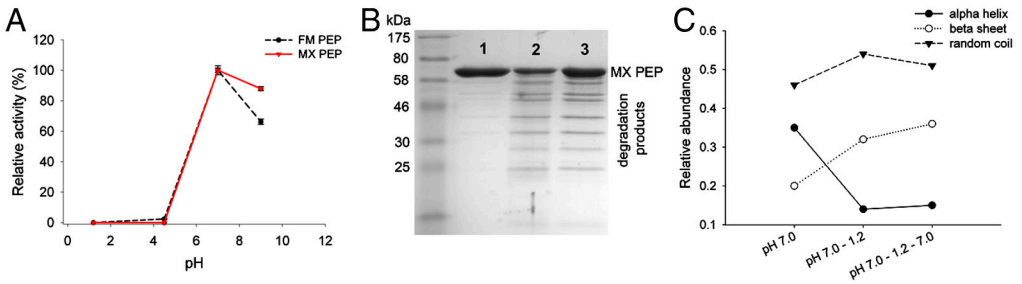


Fig. S6. Activity profiles and potential routes of inactivation of PEPs. (*A*) Activity of FM and MX PEP as function of pH measured using *Z*-Gly-Pro-*p*NA and normalized to the activity at pH 7.0 (maximum value). In vitro activity profiles of PEPs demonstrated that MX PEP was not active up to pH 7, while FM PEP showed ~2% activity at pH 4.5, Mean ± SD, $n = 3$. (*B*) SDS-PAGE of native MX PEP (lane 1) and MX PEP following an incubation at pH 1.2 [HCl/KCl solution United States Pharmacopeia (USP), lane 2] and pH 4.5 (acetate buffer USP, lane 3) for 60 min at 37 °C. SDS-PAGE was conducted using a 12.5% polyacrylamide gel under reducing (mercaptoethanol) and denaturing (heating to 95 °C) conditions. Protein load in lanes 1–3 was 3 μg. Rapid coomassie blue staining was used to visualize the protein bands. After incubation at acidic pH, additional bands corresponding to PEP degradation products could be observed. (*C*) Circular dichroism spectroscopy analysis of FM PEP (0.13 mg/mL) at pH 7.0. The pH was subsequently decreased to 1.2 using 1 M HCl and neutralized back to pH 7.0 using 1 M NaOH. Measurements were made using a Spectrometer 202 (AVIV instruments, Lakewood, NJ) with the following settings: 1 mm quartz cuvette, 25 °C, 241 – 190 nm, 0.5 nm steps, averaging 4 spectra. Raw data were converted into mean residual ellipticity (θ) and analyzed using the Dichroweb online deconvolution algorithm for protein circular dichroism spectra (1). Experiments revealed a substantial decrease in α-helical and an increase in random coil conformation at low pH which was partially recovered when neutralizing back to pH 7.0.

1 Whitmore L, Wallace BA (2004) Dichroweb, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res* 32:W668–W673.

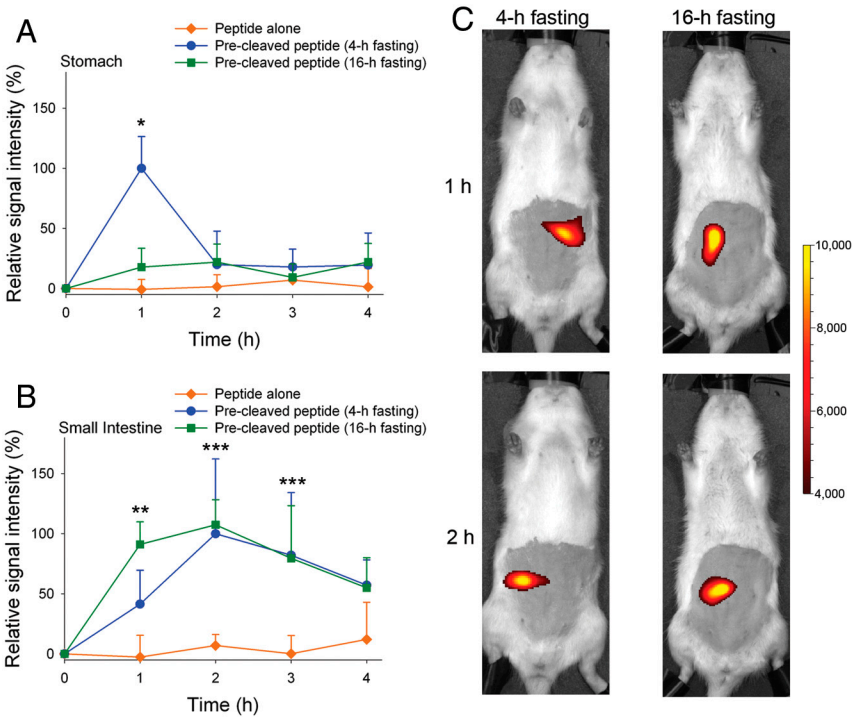


Fig. S7. Fluorescence imaging of the abdominal region as a function of fasting status. Rats were gavaged with the precleaved peptide after 4 or 16 h of fasting. (*A*, *B*) The graphs show the relative fluorescence signal in the stomach and in the small intestine following the two different fasting pretreatments. Mean + SD, $n = 4$–9. *Precleaved peptide (4-h fasting) vs. all other groups ($p < 0.01$), **Precleaved peptide (16-h fasting) vs. peptide alone ($p < 0.01$), ***Pre-cleaved peptide (4-h fasting and 16-h fasting) vs. peptide alone ($p < 0.05$). (*C*) Evolution of fluorescence signal in the GI tract. Pictures were recorded 1 and 2 h after gavage. The pictures shown are from representative rats in each set. Color scales are identical.

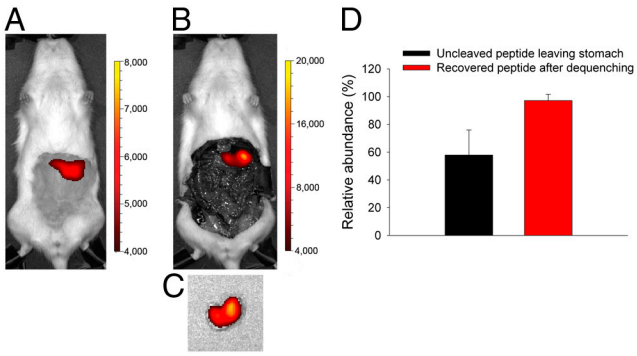


Fig. S8. Fluorescence imaging of stomach content. Rats were administered FM PEP with peptide. After 1 h, animals were killed, the abdominal wall was opened and the GI tract imaged using an IVIS® Spectrum imaging system. (*A*, *B*) The cleaved peptide could be clearly detected in the stomach 1 h after oral application in both the intact and the opened animal. (*C*) The fluorescence signal was detected in the dissected stomach. Color scales are identical for all pictures in *C* and *D*. (*D*) Residual proportion of intact peptide leaving the stomach was assessed by suspending stomach content in a mixture of phosphate buffer (10 mM) and sodium cholate (0.5%). After addition of excess FM PEP, increase in fluorescence intensity was measured and used to calculate the residual proportion of uncleaved peptide in the stomach (black bar). Subsequently, cleaved peptide was extracted using sodium cholate dissolved in acetonitrile (0.25%, w/v) and quantified by spectrofluorometry (red bar), Mean ± SD, $n = 3$.

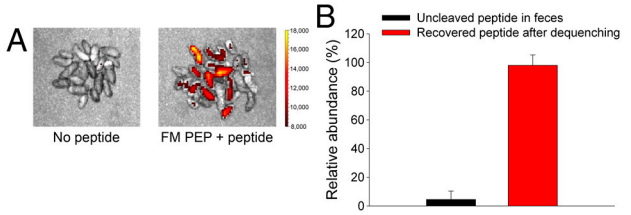


Fig. S9. Fluorescence imaging of feces. Rats were administered FM PEP with peptide. (*A*) Feces were collected 24 h after oral gavage and imaged using an IVIS® Spectrum imaging system. The cleaved peptide could be clearly detected in feces collected after oral application of FM PEP with peptide. (*B*) Residual proportion of intact peptide in the feces was assessed by suspending feces in a mixture of phosphate buffer (10 mM) and sodium cholate (0.5%). After addition of excess FM PEP, the increase in fluorescence intensity was measured and used to calculate the residual proportion of uncleaved peptide in the feces (black bar). Subsequently, cleaved peptide was extracted using sodium cholate dissolved in acetonitrile (0.25%, w/v) and quantified by spectrofluorometry. The cleaved peptide could be recovered completely from the feces indicating no systemic uptake of the peptide (red bar), Mean ± SD, $n = 3$.

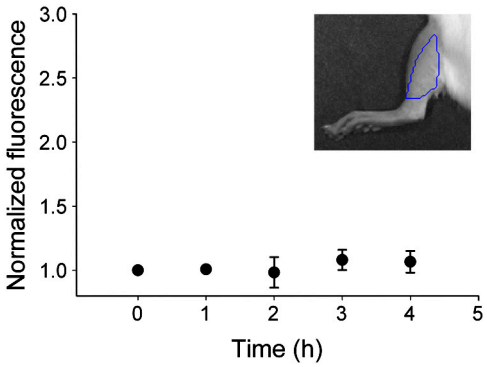
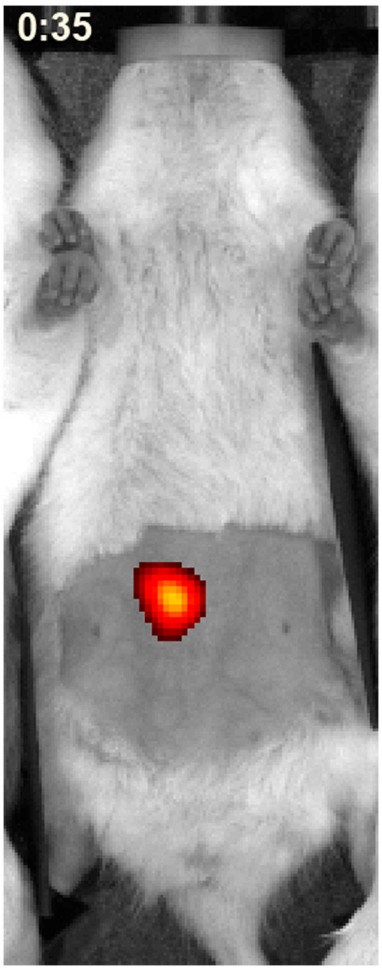


Fig. S10. Fluorescence imaging of saphenous vein region. Rats were gavaged with FM PEP followed by peptide and the fluorescence intensity in the saphenous vein region was measured (ROI indicated). This region represents a highly perfused area and thus changes in the fluorescence signal could be used to monitor peptide absorption. The graph shows the normalized fluorescence signal in the saphenous vein area (inset) during 4 h of experiment. Mean ± SD, $n = 3$. Normalized fluorescence did not change during 4 h of measurement indicating that the cleaved peptide was essentially not absorbed.



Movie S1. Dynamics of in vivo fluorescence imaging along the GI tract. The rat was fasted 8 h prior to the experiment and subsequently administered orally FM PEP with peptide. Pictures were recorded during 4 h of anesthesia. The shown time scale is h: min.

Movie S1 (WMV)

Table S1. Determination of Michaelis-Menten parameters (*A*) K_m and (*B*) k_{cat} of FM and MX PEP using E(EDANS)-LPYPQPK(Dabcyl).

E(EDANS)-LPYPQPK(Dabcyl)

	K_m (μM)		
	$t = 0$ min	$t = 10$ min	$t = 60$ min
Gastric fluid (pH 4.5)			
FM PEP	3.8 ± 0.6	n.d.	n.d.
MX PEP	3.3 ± 0.7	n.d.	n.d.
Intestinal fluid (pH 6.8)			
FM PEP	3.8 ± 0.6	4.3 ± 0.8	6.4 ± 0.5
MX PEP	3.3 ± 0.7	4.4 ± 0.7	4.9 ± 0.6
n.d. - K_m was too low to be determined in simulated gastric fluid			

	k_{cat} (s^{-1})		
	$t = 0$ min	$t = 10$ min	$t = 60$ min
Gastric fluid (pH 4.5)			
FM PEP	1.96 ± 0.50	n.d.	n.d.
MX PEP	0.42 ± 0.19	n.d.	n.d.
Intestinal fluid (pH 6.8)			
FM PEP	1.96 ± 0.50	0.25 ± 0.03	0.08 ± 0.02
MX PEP	0.42 ± 0.19	0.22 ± 0.15	0.07 ± 0.02
n.d. - k_{cat} was too low to be determined in simulated gastric fluid			

PEPs were incubated in vitro in simulated gastric (pH 4.5 with pepsin mimicking a filled stomach) and intestinal (pH 6.8 with pancreatin) fluids. At specific time points, the cleavage velocity of E(EDANS)-LPYPQPK(Dabcyl) ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 490$ nm) by PEPs was assessed by measuring the increase in fluorescence at 490 nm. Raw data were analyzed according to the Michaelis-Menten equation, and K_m and k_{cat} were calculated by Lineweaver-Burk plot. Under simulated gastric conditions at pH 4.5, FM and MX PEP lost their activity (K_m not determinable) towards the peptide within 10 min suggesting a rapid inactivation. In the intestinal fluids, PEPs retained substrate affinity (K_m unchanged for 60 min of incubation), but k_{cat} substantially decreased indicating a strongly diminished cleavage velocity.