Supporting Information

Rapid profiling of peptide stability in proteolytic environments

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The Research aricle describes a novel assay which is suitable to determine the stability of peptides in complex proteolytic environments.

The Supporting information contains the exact protocol of the solid phase peptide synthesis employed and includes an exemplary overview of synthesis yields obtained with this procedure (Table S1).

The Supporting Figures emphasize the statements of the main paper by providing additional experimental data.

Figure S1 gives the results obtained when analyzing the stability of enzyme activity in murine intestinal lavage over prolonged incubation at 37° C.

In addition to the data presented in the main paper for the enzyme trypsin (see Figure 4 A,B), concerning the determination of k_{cat}/K_M via variation of both, enzyme concentration and time, Figure S2 shows the results of the analogous experiment conducted with the enzyme chymotrypsin.

Figure S3 elaborates the results concerning the influence of the peptides' flanking regions depicted in Figure 4 C,D of the main paper by providing additional data obtained with different substrate peptides.

Table S2 contains a complete listing of the half-lifes of all 375 peptides investigated for their stability in murine intestinal fluid.

Supporting Method:

SPOT peptide synthesis on cellulose membranes[1]

Peptide libraries were SPOT-synthesized using standard fluorenylmethoxycarbonyl (Fmoc) amino acid protection chemistry. Vacuum dried Whatman 540 cellulose membranes were ester-derivatized in a closed container for 24 h at RT with Fmoc-protected proline (0.2 M Fmoc-proline, 0.25 M diisopropylcarbodiimide (DICD) and 0.46 M N-methylimidazole in deionized and desiccated dimethylformamide (DMF)). Peptide bond formation of all amino acids was performed at RT in a semiautomated cycle: Washing and incubation steps were carried out manually under agitation. (i) acetyl-blocking of reactive groups with 2 % (v/v) acetic anhydride in DMF for 24 h or 20 min (the solution was changed repeatedly), (ii) DMF-washing (1 x 30 s, 2 x 2 min), (iii) Fmoc-deprotection with 20 % (v/v) piperidine in DMF for 5 min, (iv) DMF-washing (1 x 30 s, 4 x 2 min), (v) bromophenol blue (BPB) staining with 0.01 % (w/v) BPB in DMF for 10 min (the solution was changed repeatedly) for synthesis control, (vi) ethanol-washing (1 x 30 s, 2 x 2 min), (vii) drying in the cold air flow of a hair-dryer and placement of the membrane on the tray of the pipetting device ASP 222 (Intavis AG, Cologne, Germany). (vii) Solutions of 0.2 M Fmoc-protected amino acids and 0.35 M 1-hydroxybenzotriazole (HOBt) in deionized and desiccated methyl-2-pyrrolidone (NMP) were activated with DICD (final concentration: 0.25 M) 30 min prior to each synthesis cycle. (viii) The pipetting device automatically applied 0.1 or 0.2 µl of the activated amino acid solutions onto each positionally addressed area (SPOT) on the membrane. Each amino acid application step was performed three times followed by an incubation time of at least 40 min. Proline derivatized cellulose membranes were acetyl-capped for 24 h. In the first cycle a Boc-Lys(Fmoc) amino acid solution containing 0.4 mM 5(6)-carboxytetramethylrhodamine was prepared. The rhodamine derivative rendered the SPOTs visible by eye. All SPOTs were defined by the automated application of 0.1 µl activated Boc-Lys(Fmoc) solution to the membrane resulting in the formation of a cleavable ε-lysine-proline anchor[2]. The membranes were capped for another 24 h. In the following cycles 0.2 µl activated amino acid solutions were automatically applied to the SPOTs and the time for acetyl-capping was reduced to 20 min. The first amino acid following the anchor residue was Fmoc-biocytine or Fmoc-N-γ-(N-biotinyl-3-(2-(2-(3-aminopropyloxy)-ethoxy)-ethoxy)-propyl)-L-glutamine (Merck Biosciences, Schwalbach, Germany), respectively. The latter already incorporates a PEG-moiety, whereas following biocytine amino poly(ethylene glycole) diglycolic acid (n=9) (Polypure, Oslo, Norway) was coupled. The sequence motif was synthesized in repeating cycles by adding amino acids one by one. For synthesis completion 2,4-dichlorophenoxyacetic acid (2,4-D) was applied. For side chain deprotection and diketopiperazine formation of the ε-lysine-proline anchor, the

dried membranes were incubated in a closed container for 2 x 1 h with a freshly prepared solution of 3 % triisobutylsilane, 2 % water and 50 % trifluoroacetic acid in dichloromethane (all v/v). Chemicals were washed out with dichloromethane (1 x 1 min, 3 x 10 min), a solution of 0.1 % HCl and 50 % methanol in water (1 x 1 min, 3 x 20 min) and 1 M acetic acid (1 x 1 min, 3 x 20 min) and the membranes were dried under vacuum. Visible by a tinge of rhodamine SPOTs were punched out and transferred individually to polypropylene tubes. The peptides were cleaved from the membrane by shaking overnight at 30 °C in 0.5 ml 0.1 M triethylammonium acetate and 20 % ethanol in water. Supernatants were transferred to a second tube and 0.5 ml new triethyl-ammonium acetate buffer was applied to the SPOTs for another 2 h. Supernatants were combined in the second tube and lyophilized. Peptides were dissolved in 1.5 ml L-PBS x 0.005 % (w/v) Tween 20 (L-PBST), snap frozen in liquid N_2 and stored at -80 °C.

References

(1) Frank, R. *Tetrahedron* **1992**, *48*, 9217-9232.
(2) Bray, M. B.; Maeji, N. J.; Geysen, H. M. *Tetrahedron Lett* **1990**, *31*, 5811-5814

Supporting Table 1: Synthesis yield of peptides generated by SPOT-synthesis

Random peptide	Sequence motif	SPOT synthesis amount [nmole]	Peptide amount for proteolysis experiments (3/1000 SPOT) [pmole]
1	HQEQPT	0.957 ± 0.027	2.87 ± 0.08
	AKENGMLYEFHQEQPT	0.791 ± 0.077	2.37 ± 0.23
2	VRTRSA	0.972 ± 0.093	2.92 ± 0.28
	VHNMDKPWLSVRTRSA	n.a.	n.a.
3	VWNELA	1.145 ± 0.098	3.43 ± 0.29
	WMLCRMQRFWVWNELA	n.a.	n.a.
4	AEQPAA	0.696 ± 0.040	2.09 ± 0.12
	FLHMWLLTIFAEQPAA	n.a.	n.a.
5	GDFQRT	0.921 ± 0.083	2.76 ± 0.25
	GTEKPFVEAGGDFQRT	0.502 ± 0.047	1.51 ± 0.14

Random peptides were SPOT-synthesized as described. As synthesis amounts could depend on sequence motif as well as on peptide length five different 16-mer and N-terminally shortened 6-mer peptides were synthesized with a C-terminal biotin label and an N-terminal 2,4-D-aminoundecanoic acid label. The synthesis amount was determined with a competitive ELISA. Free 2,4-D-aminoundecanoic acid (synthesized in our lab; SB, unpublished results) of known concentration was serially diluted in the peptide solution, the mixtures were transferred to an anti-2,4-D antibody-coated microtiter plate, and captured peptide was quantitated with enzyme-labeled streptavidin. From the midpoint of the resulting binding curve, representing equal amounts of competitor and peptide, the total peptide amount could be determined (geometric mean ± SE of triplicate measurements). In three cases this method was not applicable (n.a.) as some 16-mer peptides exhibited nonspecific binding to the microtiter plate.

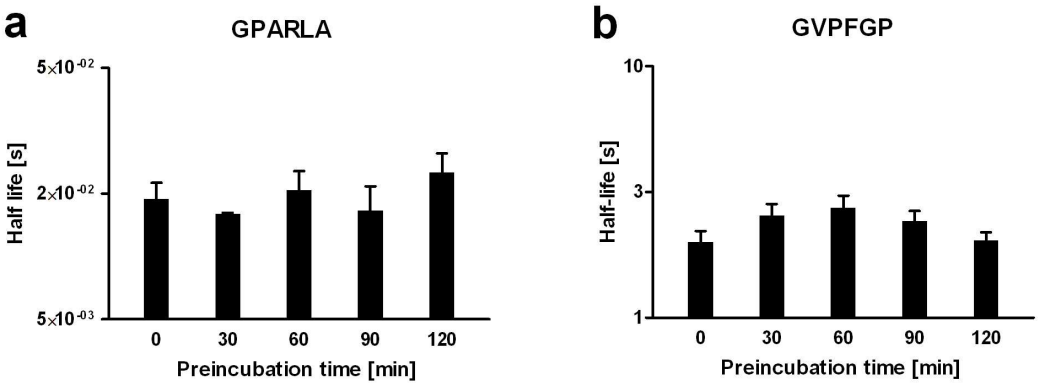

Figure S1: Stability of proteolytic activity in intestinal lavage during long incubation times. Murine small intestinal lavage was diluted in a microtiter plate to the final concentration needed for proteolysis and preincubated for 0, 30, 60, 90 or 120 min at 37° C. Immediately after the preincubation time, 67 pM peptide substrate was added and incubated for further 90 min at 37° C. After termination of the enzyme reaction, the peptide solutions were transferred to an antibody-coated microtiter plate, where uncleaved peptides were detected by enzyme-coupled signal amplification. Peptide half-lifes were calculated using equation 4. The peptides `GPARLA` (**a**) and `GVPFGP` (**b**), which contain cleavage motives for trypsin or chymotrypsin, respectively, show no significant difference in half-lifes due to incubation times up to 120 min (triplicate measurements, one-way ANOVA, Fisher's PLSD-test, $p > 0.05$).

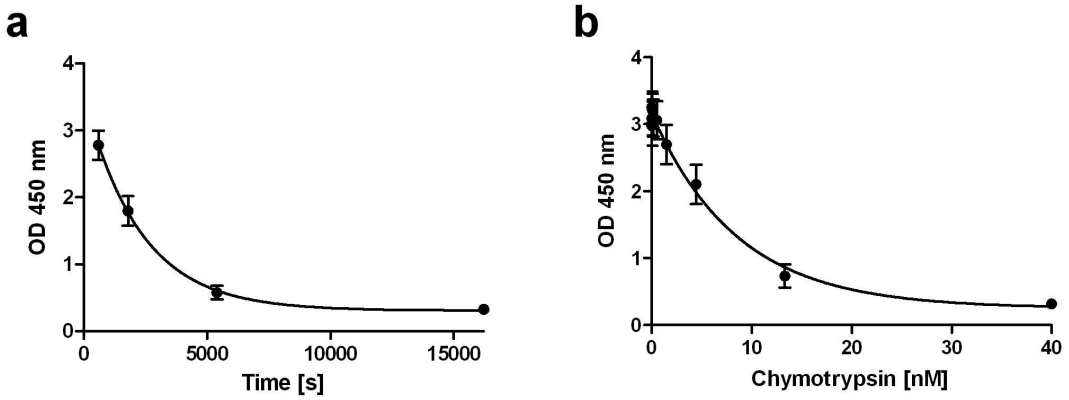

Figure S2: Determination of k_{cat}/K_M by variation of incubation time and of enzyme concentration.

Either 67 pM substrate (GVPFGP) was incubated with 2 nM chymotrypsin for 10, 30, 90 or 270 min at 37 °C (a) or chymotrypsin was serially diluted in 67 pM substrate for 90 min at 37 °C (b). After termination of the proteolysis reaction, the peptide solutions were transferred to an antibody-coated microtiter plate, where uncleaved peptides were detected by enzyme-coupled signal amplification. Plotting the course of substrate degradation against incubation time (a) or chymotrypsin concentration (b) yielded both pseudo-first order reaction kinetics and equation 2 was employed for non-linear curve-fitting. Error bars indicate the SE of fivefold measurements. k_{cat}/K_M was $1.9\times10^4 \pm 0.2 \times10^4$ M^{-1} s^{-1} (a) or $2.3\times10^4 \pm 0.4\times10^4$ M^{-1} s^{-1} (b) (geometric mean ± SE). Both values did not differ significantly from each other ($p > 0.05$, Student's two-tailed t-test).

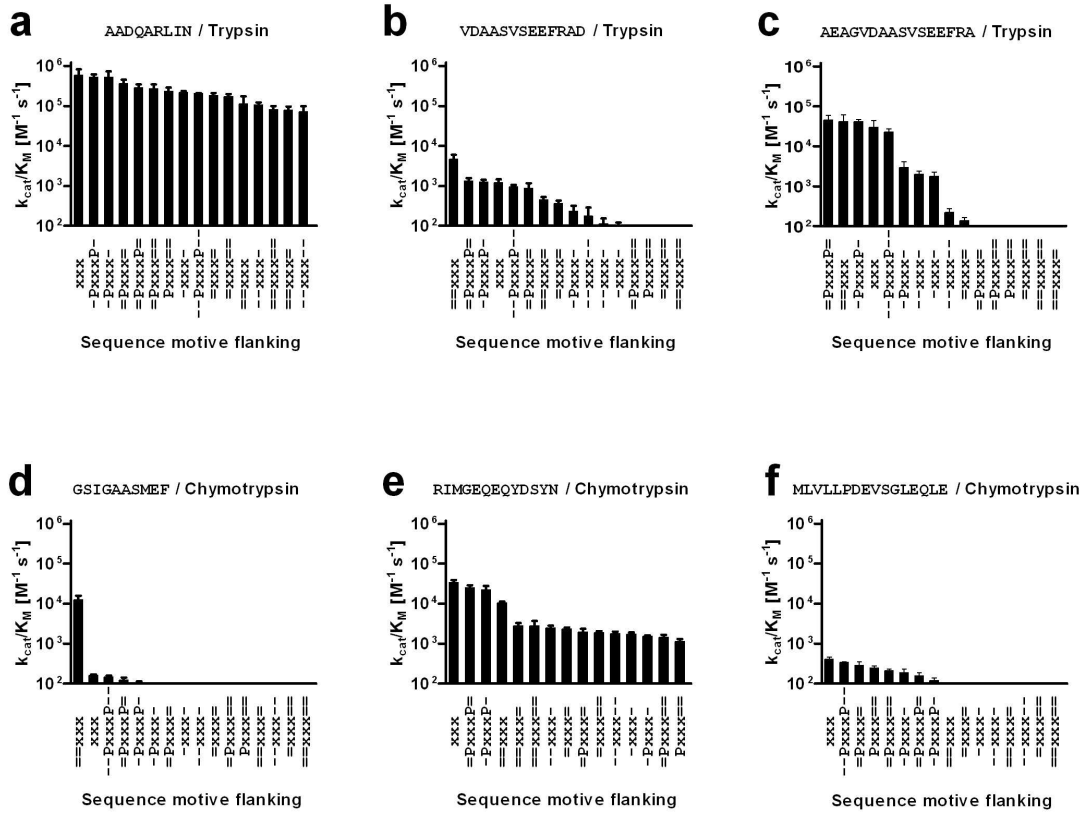

Figure S3: Influence of flanking motifs on k_{cat}/K_M.

10-mer, 13-mer and 16-mer substrate sequence motifs for trypsin (a-c) and chymotrypsin (d-f) were used with a choice of the best flanking motifs for preventing nonspecific binding to microtiter plates (Figure 3f). Chymotrypsin or trypsin were serially diluted in 67 pM substrate and incubated for 90 min at 37 °C. After termination of the enzyme reaction, the peptide solutions were transferred to an antibody-coated microtiter plate, where uncleaved peptides were detected by enzyme-coupled signal amplification. Values for k_{cat}/K_M were determined from eq. 2 after non-linear curve fitting (geometric means + SE of triplicate measurements). Figures S3c and f show the same experiment as Figure 4c and 4d in the main text. To emphasize the large difference in peptide stabilities due to different flanking motifs all y-axes have the same scaling. Direct attachment of negatively charged building blocks to the sequence motif rendered the peptides more stable to proteolysis than peptides without such flanking regions. This effect was prevented by introducing PEG between sequence motif and negatively charged building blocks; (xxx), peptide; (P), poly(ethylene glycole) diglycolic acid (n=2); (-), D-glutamate; (=), carboxyglutamate.

Table S2: Peptide stability in small intestinal fluid

	10-mer peptides	$t_{1/2}$ [s]	SE
1	GSIGAASMEF	2.7013	0.2777
2	IGAASMEFCF	0.0629	0.0168
3	AASMEFCFDV	0.0556	0.0163
4	SMEFCFDVFK	0.0103	0.0030
5	EFCFDVFKEL	0.0088	0.0014
6	CFDVFKELKV	0.0122	0.0025
7	DVFKELKVHH	0.0101	0.0018
8	FKELKVHHAN	0.0094	0.0028
9	ELKVHHANEN	0.1969	0.0086
10	KVHHANENIF	1.2897	0.0876
11	HHANENIFYC	0.0485	0.0074
12	ANENIFYCPI	0.1557	0.0240
13	ENIFYCPIAI	0.0890	0.0143
14	IFYCPIAIMS	0.0469	0.0037
15	YCPIAIMSAL	0.2911	0.0130
16	PIAIMSALAM	0.1596	0.0172
17	AIMSALAMVY	0.0512	0.0087
18	MSALAMVYLG	0.0132	0.0033
19	ALAMVYLGAK	0.0077	0.0002
20	AMVYLGAKDS	0.0111	0.0020
21	VYLGAKDSTR	0.0135	0.0009
22	LGAKDSTRTQ	0.0122	0.0006
23	AKDSTRTQIN	0.0092	0.0005
24	DSTRTQINKV	0.0210	0.0065
25	TRTQINKVVR	0.0027	0.0006
26	TQINKVVRFD	0.0094	0.0013
27	INKVVRFDKL	0.0069	0.0007
28	KVVRFDKLPG	0.0119	0.0012
29	VRFDKLPGFG	0.1526	0.0364
30	FDKLPGFGDS	25.2445	2.5943
31	KLPGFGDSIE	40.1296	7.1878
32	PGFGDSIEAQ	17.8114	2.4067
33	FGDSIEAQCG	7.1220	1.4460
34	DSIEAQCGTS	13.4815	0.0964
35	IEAQCGTSVN	6.4814	0.6663
36	AQCGTSVNVH	4.3435	0.3449
37	CGTSVNVHSS	1.1378	0.4247
38	TSVNVHSSLR	0.1177	0.0143
39	VNVHSSLRDI	0.0148	0.0038
40	VHSSLRDILN	0.0527	0.0112
41	SSLRDILNQI	0.1504	0.0112
42	LRDILNQITK	0.0939	0.0194
43	DILNQITKPN	4.1593	0.1479
44	LNQITKPNDV	20.6999	2.1888
45	QITKPNDVYS	0.2963	0.0418

	16-mer peptides	$t_{1/2}$ [s]	SE
1	GSIGAASMEFCFDVFK	0.0097	0.0025
2	IGAASMEFCFDVFKEL	0.0084	0.0020
3	AASMEFCFDVFKELKV	0.0068	0.0016
4	SMEFCFDVFKELKVHH	0.0084	0.0018
5	EFCFDVFKELKVHHAN	0.0098	0.0005
6	CFDVFKELKVHHANEN	0.0103	0.0017
7	DVFKELKVHHANENIF	0.0201	0.0039
8	FKELKVHHANENIFYC	0.0074	0.0020
9	ELKVHHANENIFYCPI	0.0851	0.0178
10	KVHHANENIFYCPIAI	0.0892	0.0174
11	HHANENIFYCPIAIMS	0.0575	0.0092
12	ANENIFYCPIAIMSAL	0.0425	0.0032
13	ENIFYCPIAIMSALAM	0.0327	0.0054
14	IFYCPIAIMSALAMVY	0.0167	0.0033
15	YCPIAIMSALAMVYLG	0.0125	0.0014
16	PIAIMSALAMVYLGAK	0.0077	0.0012
17	AIMSALAMVYLGAKDS	0.0088	0.0020
18	MSALAMVYLGAKDSTR	0.0063	0.0012
19	ALAMVYLGAKDSTRTQ	0.0045	0.0009
20	AMVYLGAKDSTRTQIN	0.0037	0.0007
21	VYLGAKDSTRTQINKV	0.0040	0.0008
22	LGAKDSTRTQINKVVR	0.0033	0.0008
23	AKDSTRTQINKVVRFD	0.0038	0.0004
24	DSTRTQINKVVRFDKL	0.0044	0.0009
25	TRTQINKVVRFDKLPG	0.0045	0.0004
26	TQINKVVRFDKLPGFG	0.0085	0.0021
27	INKVVRFDKLPGFGDS	0.0111	0.0020
28	KVVRFDKLPGFGDSIE	0.0131	0.0021
29	VRFDKLPGFGDSIEAQ	0.1209	0.0282
30	FDKLPGFGDSIEAQCG	5.6930	0.6134
31	KLPGFGDSIEAQCGTS	6.4211	0.7910
32	PGFGDSIEAQCGTSVN	5.0348	0.4914
33	FGDSIEAQCGTSVNVH	1.3951	0.1152
34	DSIEAQCGTSVNVHSS	0.4688	0.0599
35	IEAQCGTSVNVHSSLR	0.1930	0.0428
36	AQCGTSVNVHSSLRDI	0.0267	0.0019
37	CGTSVNVHSSLRDILN	0.0499	0.0040
38	TSVNVHSSLRDILNQI	0.1029	0.0019
39	VNVHSSLRDILNQITK	0.0614	0.0022
40	VHSSLRDILNQITKPN	0.1104	0.0054
41	SSLRDILNQITKPNDV	0.1963	0.0141
42	LRDILNQITKPNDVYS	0.0514	0.0085
43	DILNQITKPNDVYSFS	0.0223	0.0039
44	LNQITKPNDVYSFSLA	0.0054	0.0004
45	QITKPNDVYSFSLASR	0.0025	0.0006

	10-mer peptides	$t_{1/2}$ [s]	SE
46	TKPNDVYSFS	0.0120	0.0019
47	PNDVYSFSLA	0.0026	0.0004
48	DVYSFSLASR	0.0037	0.0005
49	YSFSLASRLY	0.0019	0.0007
50	FSLASRLYAE	0.0035	0.0009
51	LASRLYAEER	0.0031	0.0008
52	SRLYAEERYP	0.0052	0.0007
53	LYAEERYPIL	0.1052	0.0144
54	AEERYPILPE	11.4998	2.1736
55	ERYPILPEYL	1.4660	0.2397
56	YPILPEYLQC	0.1016	0.0108
57	ILPEYLQCVK	0.3817	0.0205
58	PEYLQCVKEL	0.1101	0.0190
59	YLQCVKELYR	0.0263	0.0032
60	QCVKELYRGG	0.0128	0.0019
61	VKELYRGGLE	0.0225	0.0050
62	ELYRGGLEPI	0.0244	0.0033
63	YRGGLEPINF	0.0238	0.0024
64	GGLEPINFQT	0.0336	0.0036
65	LEPINFQTAA	0.0242	0.0049
66	PINFQTAADQ	0.0812	0.0093
67	NFQTAADQAR	0.1293	0.0141
68	QTAADQAREL	0.0514	0.0126
69	AADQARELIN	0.0325	0.0061
70	DQARELINSW	0.0224	0.0025
71	ARELINSWVE	0.0156	0.0023
72	ELINSWVESQ	1.2344	0.2859
73	INSWVESQTN	3.3348	0.8046
74	SWVESQTNGI	0.7564	0.0298
75	VESQTNGIIR	0.3309	0.0104
76	SQTNGIIRNV	0.0066	0.0008
77	TNGIIRNVLQ	0.0072	0.0010
78	GIIRNVLQPS	0.0038	0.0003
79	IRNVLQPSSV	0.0049	0.0008
80	NVLQPSSVDS	0.3384	0.0684
81	LQPSSVDSQT	0.1866	0.0275
82	PSSVDSQTAM	4.5647	0.8713
83	SVDSQTAMVL	0.2013	0.0233
84	DSQTAMVLVN	0.0195	0.0030
85	QTAMVLVNAI	0.0264	0.0042
86	AMVLVNAIVF	0.0165	0.0027
87	VLVNAIVFKG	0.0087	0.0014
88	VNAIVFKGLW	0.0051	0.0008
89	AIVFKGLWEK	0.0054	0.0002
90	VFKGLWEKAF	0.0039	0.0012
91	KGLWEKAFKD	0.0053	0.0008
92	LWEKAFKDED	0.0131	0.0022
93	EKAFKDEDTQ	0.0329	0.0023

	16-mer peptides	$t_{1/2}$ [s]	SE
46	TKPNDVYSFSLASRLY	0.0011	0.0001
47	PNDVYSFSLASRLYAE	0.0014	0.0000
48	DVYSFSLASRLYAEER	0.0011	0.0002
49	YSFSLASRLYAEERYP	0.0032	0.0004
50	FSLASRLYAEERYPIL	0.0031	0.0005
51	LASRLYAEERYPILPE	0.0023	0.0003
52	SRLYAEERYPILPEYL	0.0028	0.0005
53	LYAEERYPILPEYLQC	0.0281	0.0053
54	AEERYPILPEYLQCVK	0.2682	0.0682
55	ERYPILPEYLQCVKEL	0.1018	0.0264
56	YPILPEYLQCVKELYR	0.0298	0.0047
57	ILPEYLQCVKELYRGG	0.0143	0.0021
58	PEYLQCVKELYRGGLE	0.0152	0.0013
59	YLQCVKELYRGGLEPI	0.0093	0.0007
60	QCVKELYRGGLEPINF	0.0085	0.0013
61	VKELYRGGLEPINFQT	0.0052	0.0007
62	ELYRGGLEPINFQTAA	0.0085	0.0010
63	YRGGLEPINFQTAADQ	0.0110	0.0031
64	GGLEPINFQTAADQAR	0.0207	0.0026
65	LEPINFQTAADQAREL	0.0155	0.0013
66	PINFQTAADQARELIN	0.0183	0.0015
67	NFQTAADQARELINSW	0.0247	0.0059
68	QTAADQARELINSWVE	0.0297	0.0048
69	AADQARELINSWVESQ	0.0322	0.0043
70	DQARELINSWVESQTN	0.0223	0.0047
71	ARELINSWVESQTNGI	0.0078	0.0014
72	ELINSWVESQTNGIIR	0.1455	0.0179
73	INSWVESQTNGIIRNV	0.0095	0.0028
74	SWVESQTNGIIRNVLQ	0.0099	0.0027
75	VESQTNGIIRNVLQPS	0.0085	0.0025
76	SQTNGIIRNVLQPSSV	0.0032	0.0008
77	TNGIIRNVLQPSSVDS	0.0033	0.0003
78	GIIRNVLQPSSVDSQT	0.0050	0.0013
79	IRNVLQPSSVDSQTAM	0.0069	0.0022
80	NVLQPSSVDSQTAMVL	0.1618	0.0189
81	LQPSSVDSQTAMVLVN	0.0332	0.0044
82	PSSVDSQTAMVLVNAI	0.0371	0.0060
83	SVDSQTAMVLVNAIVF	0.0236	0.0047
84	DSQTAMVLVNAIVFKG	0.0080	0.0013
85	QTAMVLVNAIVFKGLW	0.0051	0.0003
86	AMVLVNAIVFKGLWEK	0.0061	0.0004
87	VLVNAIVFKGLWEKAF	0.0030	0.0008
88	VNAIVFKGLWEKAFKD	0.0028	0.0009
89	AIVFKGLWEKAFKDED	0.0037	0.0001
90	VFKGLWEKAFKDEDTQ	0.0057	0.0008
91	KGLWEKAFKDEDTQAM	0.0084	0.0025
92	LWEKAFKDEDTQAMPF	0.0101	0.0015
93	EKAFKDEDTQAMPFRV	0.0037	0.0005

	10-mer peptides	$t_{1/2}$ [s]	SE
94	AFKDEDTQAM	7.6573	2.8082
95	KDEDTQAMPF	0.6468	0.0314
96	EDTQAMPFRV	0.0033	0.0001
97	TQAMPFRVTE	0.0047	0.0012
98	AMPFRVTEQE	0.0063	0.0002
99	PFRVTEQESK	0.1310	0.0214
100	RVTEQESKPV	2.1948	0.2307
101	TEQESKPVQM	19.4782	3.0864
102	QESKPVQMMY	3.8079	0.5193
103	SKPVQMMYQI	0.1240	0.0080
104	PVQMMYQIGL	0.0880	0.0082
105	QMMYQIGLFR	0.0060	0.0010
106	MYQIGLFRVA	0.0025	0.0006
107	QIGLFRVASM	0.0015	0.0001
108	GLFRVASMAS	0.0060	0.0018
109	FRVASMASEK	0.0082	0.0021
110	VASMASEKMK	0.1745	0.0345
111	SMASEKMKIL	0.0072	0.0017
112	ASEKMKILEL	0.0103	0.0027
113	EKMKILELPF	0.0135	0.0039
114	MKILELPFAS	0.0183	0.0051
115	ILELPFASGT	0.0767	0.0173
116	ELPFASGTMS	0.0648	0.0104
117	PFASGTMSML	3.8440	1.2250
118	ASGTMSMLVL	0.1692	0.0097
119	GTMSMLVLLP	0.0949	0.0108
120	MSMLVLLPDE	0.0317	0.0056
121	MLVLLPDEVS	0.3230	0.0694
122	VLLPDEVSGL	8.3587	0.5968
123	LPDEVSGLEQ	0.3722	0.1025
124	DEVSGLEQLE	0.1719	0.0223
125	VSGLEQLESI	1.0014	0.0596
126	GLEQLESIIN	0.5162	0.1333
127	EQLESIINFE	0.0686	0.0208
128	LESIINFEKL	0.0663	0.0119
129	SIINFEKLTE	0.0667	0.0179
130	INFEKLTEWT	0.0541	0.0065
131	FEKLTEWTSS	0.0563	0.0088
132	KLTEWTSSNV	0.4176	0.1064
133	TEWTSSNVME	0.5842	0.1034
134	WTSSNVMEER	0.9286	0.0851
135	SSNVMEERKI	0.0399	0.0119
136	NVMEERKIKV	0.0072	0.0002
137	MEERKIKVYL	0.0037	0.0011
138	ERKIKVYLPR	0.0008	0.0002
139	KIKVYLPRMK	0.0017	0.0005
140	KVYLPRMKME	0.0044	0.0012
141	YLPRMKMEEK	0.0088	0.0033

	16-mer peptides	$t_{1/2}$ [s]	SE
94	AFKDEDTQAMPFRVTE	0.0047	0.0015
95	KDEDTQAMPFRVTEQE	0.0049	0.0003
96	EDTQAMPFRVTEQESK	0.0041	0.0009
97	TQAMPFRVTEQESKPV	0.0076	0.0012
98	AMPFRVTEQESKPVQM	0.0050	0.0012
99	PFRVTEQESKPVQMMY	0.1097	0.0086
100	RVTEQESKPVQMMYQI	0.1086	0.0146
101	TEQESKPVQMMYQIGL	0.0696	0.0035
102	QESKPVQMMYQIGLFR	0.0055	0.0013
103	SKPVQMMYQIGLFRVA	0.0020	0.0001
104	PVQMMYQIGLFRVASM	0.0022	0.0005
105	QMMYQIGLFRVASMAS	0.0021	0.0002
106	MYQIGLFRVASMASEK	0.0025	0.0001
107	QIGLFRVASMASEKMK	0.0021	0.0004
108	GLFRVASMASEKMKIL	0.0047	0.0002
109	FRVASMASEKMKILEL	0.0026	0.0002
110	VASMASEKMKILELPF	0.0075	0.0029
111	SMASEKMKILELPFAS	0.0073	0.0023
112	ASEKMKILELPFASGT	0.0034	0.0016
113	EKMKILELPFASGTMS	0.0134	0.0018
114	MKILELPFASGTMSML	0.0220	0.0052
115	ILELPFASGTMSMLVL	0.0507	0.0089
116	ELPFASGTMSMLVLLP	0.0410	0.0096
117	PFASGTMSMLVLLPDE	0.0444	0.0057
118	ASGTMSMLVLLPDEVS	0.0325	0.0024
119	GTMSMLVLLPDEVSGL	0.0306	0.0103
120	MSMLVLLPDEVSGLEQ	0.0139	0.0025
121	MLVLLPDEVSGLEQLE	0.1049	0.0418
122	VLLPDEVSGLEQLESI	0.2344	0.0626
123	LPDEVSGLEQLESIIN	0.1597	0.0278
124	DEVSGLEQLESIINFE	0.0265	0.0024
125	VSGLEQLESIINFEKL	0.0393	0.0053
126	GLEQLESIINFEKLTE	0.0228	0.0044
127	EQLESIINFEKLTEWT	0.0345	0.0092
128	LESIINFEKLTEWTSS	0.0424	0.0057
129	SIINFEKLTEWTSSNV	0.0653	0.0089
130	INFEKLTEWTSSNVME	0.0497	0.0079
131	FEKLTEWTSSNVMEER	0.0286	0.0062
132	KLTEWTSSNVMEERKI	0.0414	0.0098
133	TEWTSSNVMEERKIKV	0.0139	0.0035
134	WTSSNVMEERKIKVYL	0.0060	0.0024
135	SSNVMEERKIKVYLPR	0.0021	0.0004
136	NVMEERKIKVYLPRMK	0.0021	0.0003
137	MEERKIKVYLPRMKME	0.0014	0.0007
138	ERKIKVYLPRMKMEEK	0.0012	0.0005
139	KIKVYLPRMKMEEKYN	0.0016	0.0002
140	KVYLPRMKMEEKYNLT	0.0030	0.0008
141	YLPRMKMEEKYNLTSV	0.0054	0.0014

	10-mer peptides	$t_{1/2}$ [s]	SE
142	PRMKMEEKYN	0.0056	0.0010
143	MKMEEKYNLT	0.0367	0.0073
144	MEEKYNLTSV	0.0093	0.0020
145	EKYNLTSVLM	0.0124	0.0013
146	YNLTSVLMAM	0.0294	0.0077
147	LTSVLMAMGI	0.0787	0.0218
148	SVLMAMGITD	0.1194	0.0217
149	LMAMGITDVF	0.8333	0.0550
150	AMGITDVFSS	0.0154	0.0024
151	GITDVFSSSA	0.0178	0.0041
152	TDVFSSSANL	0.0190	0.0021
153	VFSSSANLSG	0.1849	0.0306
154	SSSANLSGIS	0.7012	0.0776
155	SANLSGISSA	0.6149	0.1038
156	NLSGISSAES	1.5216	0.1626
157	SGISSAESLK	0.5455	0.1247
158	ISSAESLKIS	0.0401	0.0052
159	SAESLKISQA	0.0451	0.0095
160	ESLKISQAVH	0.0465	0.0095
161	LKISQAVHAA	0.0192	0.0040
162	ISQAVHAAHA	0.1691	0.0111
163	QAVHAAHAEI	0.1777	0.0249
164	VHAAHAEINE	0.1356	0.0201
165	AAHAEINEAG	0.7772	0.0871
166	HAEINEAGRE	0.1388	0.0195
167	EINEAGREVV	0.1025	0.0148
168	NEAGREVVGS	0.1284	0.0033
169	AGREVVGSAE	0.0967	0.0130
170	REVVGSAEAG	1.7169	0.3093
171	VVGSAEAGVD	4.0618	0.7241
172	GSAEAGVDAA	3.1271	0.3753
173	AEAGVDAASV	0.0711	0.0070
174	AGVDAASVSE	0.0548	0.0059
175	VDAASVSEEF	0.0426	0.0043
176	AASVSEEFRA	0.0246	0.0036
177	SVSEEFRADH	0.0788	0.0120
178	SEEFRADHPF	0.0759	0.0053
179	EFRADHPFLF	0.0569	0.0077
180	RADHPFLFCI	0.0056	0.0011
181	DHPFLFCIKH	0.0042	0.0010
182	PFLFCIKHIA	0.0046	0.0009
183	LFCIKHIATN	0.0185	0.0012
184	CIKHIATNAV	0.0929	0.0059
185	KHIATNAVLF	0.0719	0.0032
186	IATNAVLFFG	0.0048	0.0007
187	TNAVLFFGRC	0.0015	0.0005
188	AVLFFGRCVS	0.0012	0.0000
189	VLFFGRCVSP	0.0014	0.0002

	16-mer peptides	$t_{1/2}$ [s]	SE
142	PRMKMEEKYNLTSVLM	0.0051	0.0011
143	MKMEEKYNLTSVLMAM	0.0321	0.0026
144	MEEKYNLTSVLMAMGI	0.0225	0.0024
145	EKYNLTSVLMAMGITD	0.0211	0.0013
146	YNLTSVLMAMGITDVF	0.0298	0.0038
147	LTSVLMAMGITDVFSS	0.0170	0.0011
148	SVLMAMGITDVFSSSA	0.0276	0.0016
149	LMAMGITDVFSSSANL	0.0241	0.0012
150	AMGITDVFSSSANLSG	0.0262	0.0020
151	GITDVFSSSANLSGIS	0.0240	0.0043
152	TDVFSSSANLSGISSA	0.0361	0.0031
153	VFSSSANLSGISSAES	0.2471	0.0395
154	SSSANLSGISSAESLK	0.3682	0.0067
155	SANLSGISSAESLKIS	0.0601	0.0051
156	NLSGISSAESLKISQA	0.0601	0.0050
157	SGISSAESLKISQAVH	0.0526	0.0063
158	ISSAESLKISQAVHAA	0.0502	0.0090
159	SAESLKISQAVHAAHA	0.0475	0.0035
160	ESLKISQAVHAAHAEI	0.0463	0.0038
161	LKISQAVHAAHAEINE	0.0154	0.0011
162	ISQAVHAAHAEINEAG	0.1789	0.0120
163	QAVHAAHAEINEAGRE	0.0901	0.0103
164	VHAAHAEINEAGREVV	0.0783	0.0052
165	AAHAEINEAGREVVGS	0.1269	0.0070
166	HAEINEAGREVVGSAE	0.1531	0.0126
167	EINEAGREVVGSAEAG	0.1950	0.0348
168	NEAGREVVGSAEAGVD	0.1676	0.0473
169	AGREVVGSAEAGVDAA	0.1212	0.0155
170	REVVGSAEAGVDAASV	0.0694	0.0112
171	VVGSAEAGVDAASVSE	0.0475	0.0109
172	GSAEAGVDAASVSEEF	0.0261	0.0050
173	AEAGVDAASVSEEFRA	0.0214	0.0036
174	AGVDAASVSEEFRADH	0.0322	0.0064
175	VDAASVSEEFRADHPF	0.0389	0.0107
176	AASVSEEFRADHPFLF	0.0233	0.0051
177	SVSEEFRADHPFLFCI	0.0029	0.0006
178	SEEFRADHPFLFCIKH	0.0038	0.0010
179	EFRADHPFLFCIKHIA	0.0031	0.0005
180	RADHPFLFCIKHIATN	0.0038	0.0003
181	DHPFLFCIKHIATNAV	0.0046	0.0010
182	PFLFCIKHIATNAVLF	0.0055	0.0012
183	LFCIKHIATNAVLFFG	0.0038	0.0009
184	CIKHIATNAVLFFGRC	0.0025	0.0003
185	KHIATNAVLFFGRCVS	0.0008	0.0001
186	HIATNAVLFFGRCVSP	0.0009	0.0002

Half-lifes of 10-mer (left side) and 16-mer (right side) ovalbumin peptides (nested peptides covering the entire ovalbumin sequence with a frame shift of two amino acids) in small intestinal fluid were measured using the optimized peptide construct described in Figure 4E of the main paper and murine intestinal lavage of known dilution. Calculated half-lifes were extrapolated to undiluted enzyme solutions. Data represent the geometric mean and SE of triplicate measurements.