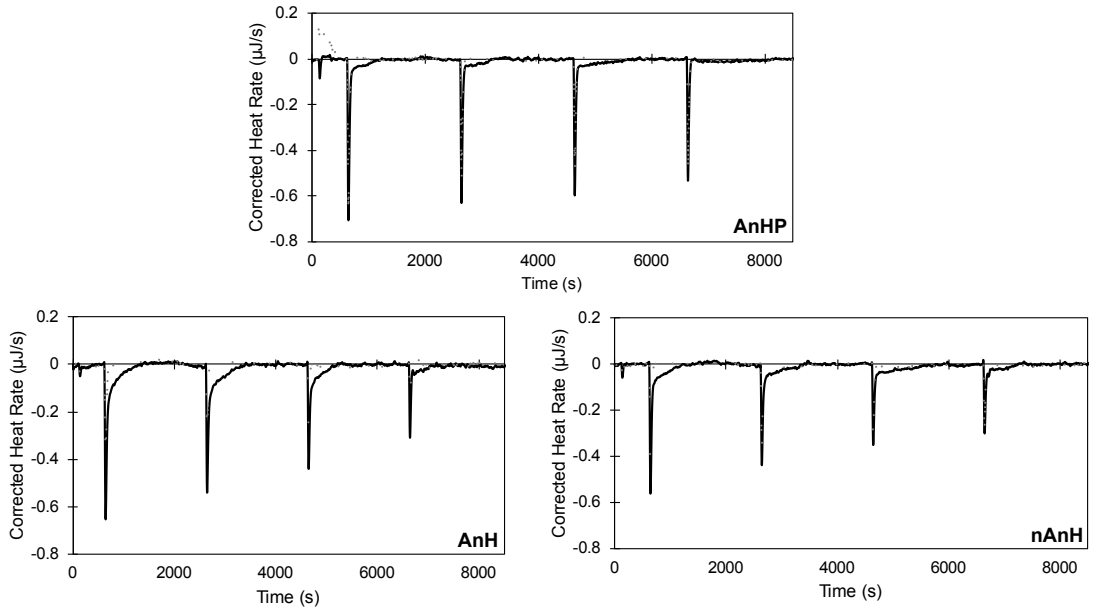
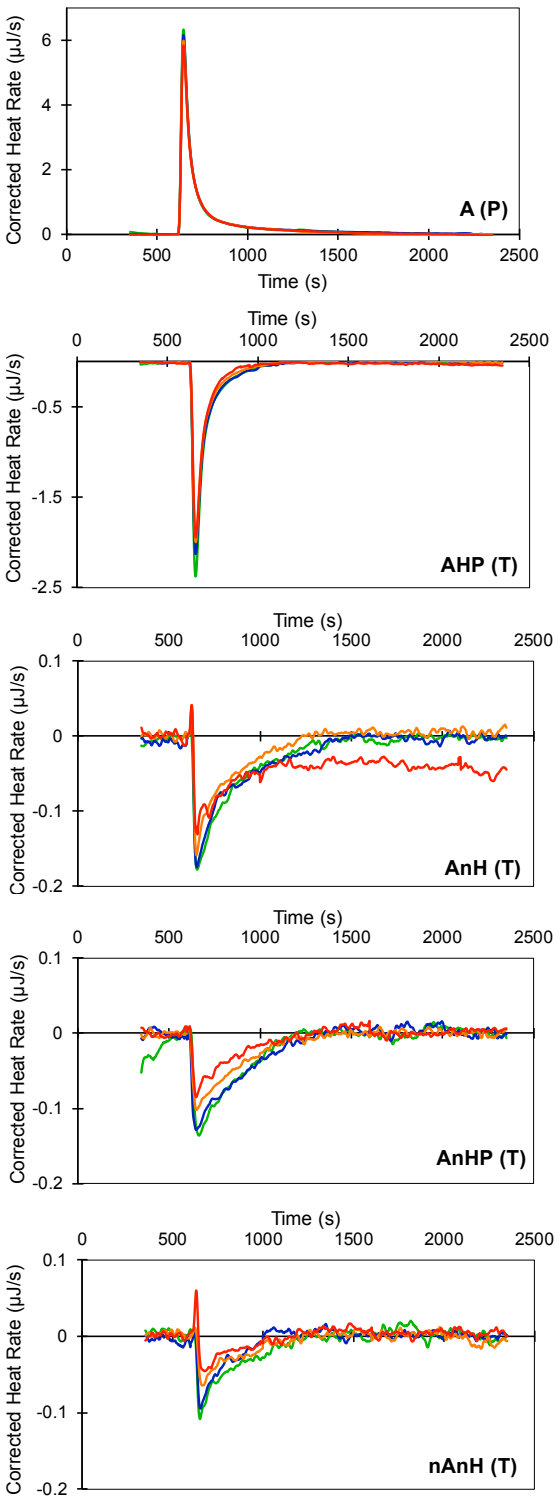
Electronic Supplementary Material (ESI) for Food & Function.

Supplementary material

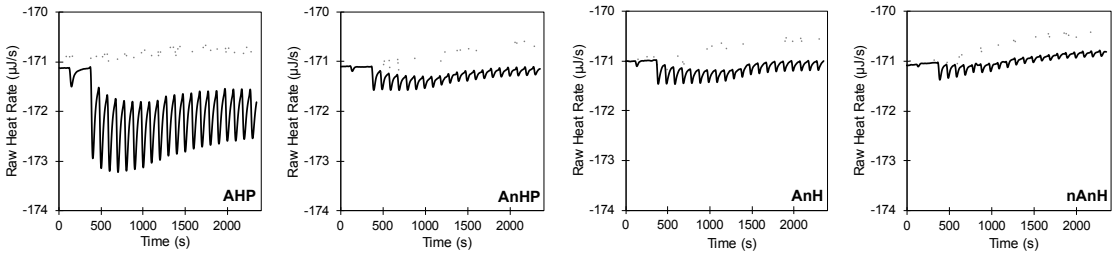


S 1. Continuous injection assay. Exemplar of calorimetric rate measurements of the blank injection of 100 µM substrate into phosphate buffer (dotted line) and reaction injection of substrate into 2.5 µM trypsin (solid line). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell. Acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH),

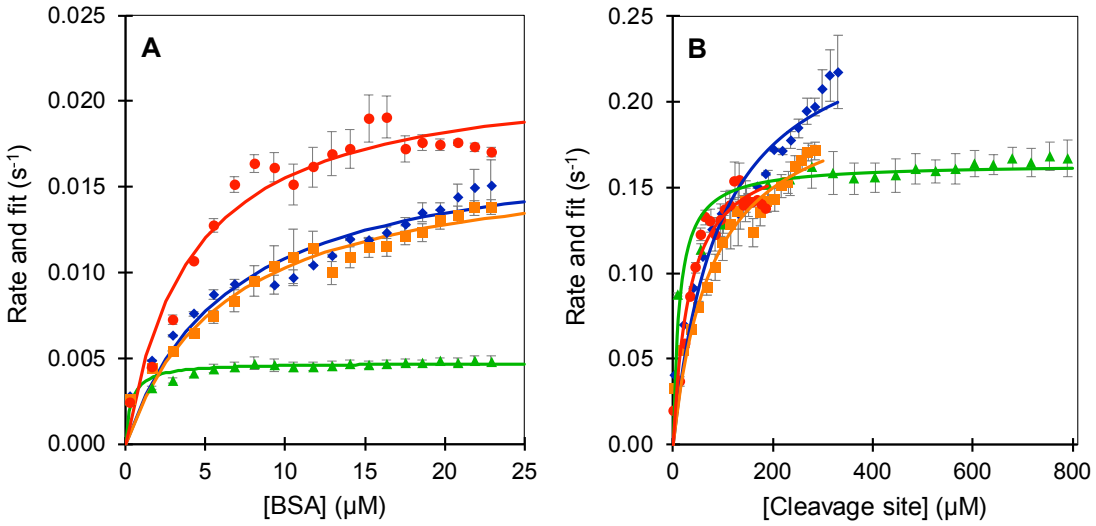


S 2. Superimposed heat rates from the continuous injection assay BSA at pH 2 (A) injected to a pepsin solution. BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH), injected into a trypsin solution at pH 8. (1st injection green, 2nd blue, 3rd orange, 4th red).

Just as in the continuous injection assay, the higher amount of substrate that is injected results in a higher signal intensity of heat rate, this is the case of BSA injected into pepsin and the pepsin-hydrolysate into trypsin (S 3). Contrarily, the lower amount of cleavage sites for trypsin in the intact proteins result in a low signal intensity, in such cases the instrument drift is more noticeable than for larger signal intensities [1]. This might explain the greater error associated to the rate estimates at high substrate concentration of trypsin-catalysed hydrolysis of intact protein, especially when it has not been previously acidified (Figure 5).



S 3. Multiple injection assay of trypsin-catalysed hydrolysis at pH 8. Exemplar of calorimetric rate measurements of the blank injection of 100 µM substrate into phosphate buffer (dotted line) and reaction injection of substrate into 2.5 µM trypsin (solid line). BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell.



S 4. Reaction rate of trypsin-catalysed hydrolysis at pH 8 of BSA pepsin-hydrolysate (triangle), acidified, non-hydrolysed BSA with inactive pepsin (diamond), acidified, non-hydrolysed BSA (square) and non-acidified, non-hydrolysed BSA (circle). Error bars represent standard deviation from three replicate estimations. Fit to the Michaelis Menten model (line). Protein (A) and cleavage sites (B) considered as substrates, rates estimated from the appropriate ΔHapp (kJ/ mol BSA or kJ/ mol cleavage sites).

Kinetic parameters of pepsin and trypsin-catalysed hydrolysis of peptide bonds

S 5. Kinetic parameters of pepsin-catalysed hydrolysis of small synthetic peptides and derivatives at pH 2 – 2.2. Selected, representative values from the references are included. Parameters are ranked according to their specificity constant (kcat/km). Parameters estimated in this study for BSA are highlighted.

Substrate	**K_m (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (1/s mM)**	Reference
Ac-F-FNH_2	-	-	0.02	[2]
Ac-F-$Y(NO_2)_2$	0.50	0.011	0.02	[3]
Ac-F-Y-OEt	0.94	0.021	0.02	[4]
Ac-F-F	1.40	0.04	0.03	[3]
Ac-$Y(NO_2)_2$-F	0.41	0.06	0.13	[3]
Z-F-F-G-OMe3P	0.80	0.18	0.23	[5]
Ac-F-F-G	1.7	0.39	0.23	[3]
BSA A	0.78	0.23	0.30	
Z-H-F-F-OEt	0.33	0.11	0.33	[4]
Z-F-F-OP4P	0.71	0.49	0.69	[5]
Z-F(NO_2)-F-OP4P	0.50	0.69	1.4	[5]
Z-G-G-F(NO_2)-F-OP4P	1.10	8.1	7.4	[5]
BSA	0.200	4.44	22.1	[6]
Z-G-G-F-F-OP4P	0.80	56.5	70.6	[5]

S 6. Kinetic parameters of trypsin-catalysed hydrolysis of small synthetic peptides and amino acid derivatives at pH 8. Selected, representative values from the references are included. Parameters are ranked according to their specificity constant (kcat/km). Substrates of deamidation are presented in italics. Parameters estimated in this study for BSA are highlighted.

Substrate	**K_m (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (1/s mM)**	Reference
Z-R-V-L-Nan	0.20	0.001	0.005	[7]
R-V-L-Nan	0.10	0.007	0.070	[7]
BACA	4.30	0.33	0.077	[8]
Ac-R-V-OMe	6.69	2.2	0.33	[9]
BLA	4.60	1.9	0.4	[8]
BANA	0.14	0.08	0.6	[10]
H-G-P-R-V-OH	1.20	1.2	1.0	[9]
BAA	2.50	2.8	1.1	[8]
Ac-R-G-OEt	3.00	4.2	1.4	[9]
BSA AnHP	0.08	0.21	2.6	
BSA AnH	0.09	0.26	2.9	
M-R-F-A	1.90	7.1	3.7	[11]
BSA nAnH	0.03	0.18	5.4	
Ac-G-R-V-OMe	20.00	3.6	0.2	[9]
Ac-G-G-R-G-OEt	2.90	19.0	6.6	[9]
Ac-V-R-G-P-R-OH	0.37	4.2	11.4	[9]
F-M-R-F(NH2)	5.90	69.0	11.7	[11]
BSA AHP	0.01	0.16	12.5	
Ac-E-G-G-G-G-R-G-OEt	0.34	5.6	16.5	[9]
F-R-S-V	4.80	99.2	20.7	[11]
Bz-V-L-K-Nan	0.40	8.99	22.5	[7]
Ac-G-P-R-V-OEt	4.70	290.0	61.7	[9]
Ac-P-R-V-OMe	1.50	200.0	133.3	[9]
Bz-F-F-R-Nan	0.06	12.8	198.4	[7]
Ac-E-V-R-G-OMe	1.30	290.0	223.1	[9]
Bz-F-T-R-Nan	0.05	24.8	488.6	[7]
I-P-R-Nan	0.01	69.8	4976.7	[7]

Ac, Acetyl; BAA, N_α-Benzoyl-L-argininamide; BACA, N_α-benzoyl-S-2-aminoethyl-L-cysteinamide; BANA, N_α-Benzoyl-DL-arginine β-naphthylamide; BLA, N_α-benzoyl-L-lysinamide; Bz, benzoyl; Nan, p-nitroanilide; OP4P, 3-(4-pyridyl)propyl-1-oxy; P, pyridyl; Z, benzyloxycarbonyl.

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