

Supplementary Information

Screening of α -amylase/trypsin inhibitor activity in wheat, spelt and einkorn by high-performance thin-layer chromatography

Isabel Müller, Bianca Schmid, Loredana Bosa, Gertrud Elisabeth Morlock*

Chair of Food Science, Institute of Nutritional Science, and Interdisciplinary Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

*Corresponding author: Prof. Dr. Gertrud Morlock, phone: +49-641-9939141; fax +49-641-99-39149, email: gertrud.morlock@uni-giessen.de

Table of contents

Table S1	Absorbance values of the three saccharides released after the amylolysis for the different flour extracts, the positive controls (PC1/PC2, acarbose) and the negative control (NC, bi-distilled water) as well as the corrected absorbance (flour extract impurities subtracted), relative inhibition regarding each saccharide and the overall inhibition referred to the NC.	S4
Fig. S1	HPTLC-Vis chromatograms for mobile phase optimization with different ratios of 2-butanol/ammonia (25%)/pyridine/water 1) 10:5:17:13, 2) 19:5:8:13, 3) 19:5:17:6, 4) 19:5:17:13(V/V/V/V); HPTLC silica gel 60 plate derivatized with the ninhydrin reagent and detected at white light illumination (remission and transmission mode).	S5
Fig. S2	HPTLC-Vis chromatograms showing separated refined wheat extract (5 µL/band) with different incubation times (0–30 min) of the amylolysis using either iodine/potassium iodide or iodine vapour as derivatization for detection of α-amylase inhibition in comparison with derivatization with the ninhydrin reagent for proteins and the diphenylamine aniline phosphoric acid reagent (DPA) for saccharides. As a negative control (NC), the assay was performed without enzyme. HPTLC silica gel 60 plates developed with 2-butanol/ammonia (25%)/pyridine/water 19:5:17:6 (V/V/V/V) up to 65 mm and documented at white light illumination (remission and transmission mode).	S6
Fig. S3	HPTLC-Vis chromatograms showing saccharide evaluation of the crude and filtered refined wheat extract (5 µL/band) and the corresponding filtrate (10 µL/band). The wheat extract was filtered once or twice for 30 min with a 3k Da Amicon Ultra-0,5 filter. For comparison, reference standards (1 µg/band) glucose (Glc), maltose (Mal) and maltotriose (Mal3) were applied on HPTLC silica gel 60 plates, developed with acetonitrile/water/2-propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the diphenylamine aniline phosphoric acid reagent and documented at white light illumination (remission and transmission mode).	S7
Fig. S4	NP-HPTLC-nanoGIT chromatogram (amylolysis with 45.5 mU/band α-amylase + 2 µg/band soluble starch) after both on-surface pre- and main incubation of the positive control acarbose (PC , 5 µg/band) and the negative control bi-distilled water (NC). For comparison, reference standards (1 µg/band) of glucose (Glc), maltose (Mal) and maltotriose (Mal3) were applied on HPTLC silica gel 60 plates, developed with acetonitrile/water/2-propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the <i>p</i> -aminobenzoic acid reagent and documented at FLD 366 nm.	S8
Fig. S5	Evaluation of an appropriate acarbose concentration as the positive control (PC) with an in-vial pre-incubation (30 min) and on-surface incubation (60 min). The pre-incubated enzyme-inhibitor mixture (11 µL/band) contained either acarbose (0.4 µg/µL) + α-amylase (4.5 mU/µL) in 25.1 µL (25:100), acarbose (80 ng/µL) + α-amylase (4.5 mU/µL) in 25.1 µL	S9

	(124:100) or acarbose (2.7 ng/μL) + α-amylase (10.3 mU/μL) in 11 μL (83:1). The negative control (NC) was α-amylase (113.8 mU/μL) + bi-distilled water. All samples were overspotted with soluble starch (2 μg/μL). For comparison, reference standards of glucose (Glc) 0.5 μg/μL, maltose (Mal) and maltotriose (Mal3) each 1 μg/μL were applied on HPTLC silica gel 60 plates, developed with acetonitrile/water/2-propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the <i>p</i> -aminobenzoic acid reagent and documented at FLD 366 nm.	
Fig. S6	NP-HPTLC-nanoGIT (proteolysis)-Vis chromatogram of different ratios of trypsin to tryptone (1:1–2:100), trypsin (3 μg/μL) and tryptone (5 μg/μL). For the trypsin assay, tryptone (5 μg/μL) was overspotted with different volumes (0.4–18.5 μL) of 0.27 μg/μL trypsin. The HPTLC silica gel 60 plate was developed with 2-butanol/ammonia (25%)/pyridine/water 19:5:17:13 (V/V/V/V) up to 70 mm, derivatized with the ninhydrin reagent and documented at white light illumination in remission-transmission mode.	S10
Fig. S7	RP-HPTLC-nanoGIT (proteolysis)-Vis chromatogram of the influence on the peptide separation and derivatization of an acidic or basic mobile phase on HPTLC RP-18 W plates (non-)buffered with citrate-phosphate buffer (pH 12). The trypsin assay (5 μL/μL, tryptone overspotted with trypsin) were applied with a trypsin/tryptone ratio of 1:20 (c₁) or 1:100 (c₂). Tryptone (a , 5 μg/μL) and trypsin (b , 0.25 μg/μL) were applied beside. The plates were developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) or 2-propanol/ammonia (25%)/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the ninhydrin reagent and documented at white light illumination in remission-transmission mode.	S11
Fig. S8	RP-HPTLC-nanoGIT (proteolysis)-FLD chromatogram showing the influence of the enzyme-substrate ratio (1:20 or 1:100) and incubation time (2 or 18 h) on the in-vial trypsin-casein assay and the application volume (5 or 10 μL) on the peptide separation on HPTLC RP-18 W plates. The in-vial trypsin-casein assay was 100 μL casein (1 μg/μL) with trypsin (0.33 μg/μL) in the ratio of 1:20 or 1:100. The plate was developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the fluorescamine reagent and documented at FLD 366 nm in enhanced mode.	S12
Fig. S9	RP-HPTLC-nanoGIT (proteolysis)-FLD chromatogram of the influence of pre-incubation times of 15 min (a) or 30 min (b) with different volumes of wheat extract (7, 17 and 27 μL) or a trypsin inhibitor as positive control (PC) on the in-vial trypsin inhibition assay. Application of 10 μL/μL of each wheat assay, the PC and the negative control (NC , trypsin-casein assay (1:100) and a wheat extract blank (27 μL) on HPTLC RP-18 W plates. The plate was developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the fluorescamine reagent and documented at FLD 366 nm in enhanced mode.	S13

Table S1

Absorbance values of the three saccharides released after the amylolysis for the different flour extracts ($n = 1$), the positive controls (PC1/PC2, acarbose) and the negative control (NC, bi-distilled water) as well as the corrected absorbance (flour extract impurities subtracted), relative inhibition regarding each saccharide and the overall inhibition referred to the NC.

	Saccharide	Absorbance	Corrected absorbance	Inhibition [%]	Overall inhibition [%]
Wheat extract blank	Glucose	0.05654444	Blank		
	Maltose	0.06809270			
	Maltotriose	0.00841666			
Wheat extract assay	Glucose	0.09134534	0.034800903	-59	-40
	Maltose	0.21398657	0.145893872	-119	
	Maltotriose	0.03206499	0.023648334	59	
Whole wheat extract blank	Glucose	0.04721251	Blank		
	Maltose	0.05110990			
	Maltotriose	0.00931743			
Whole wheat extract assay	Glucose	0.06811189	0.020899378	4	-16
	Maltose	0.18340296	0.132293060	-98	
	Maltotriose	0.02511425	0.015796815	73	
Einkorn extract blank	Glucose	0.05547531	Blank		
	Maltose	0.02432215			
Einkorn extract assay	Glucose	0.08699152	0.031516217	-44	-14
	Maltose	0.14527881	0.120956658	-81	
	Maltotriose	0.01343024	-	77	
Spelt extract blank	Glucose	0.02496919	Blank		
	Maltose	0.02618494			
Spelt extract assay	Glucose	0.03958507	0.014615879	33	15
	Maltose	0.12393922	0.097754274	-47	
	Maltotriose	0.01138892	-	80	
Whole spelt extract blank	Glucose	0.07248845	Blank		
	Maltose	0.06095526			
	Maltotriose	0.01227069			
Whole spelt extract assay	Glucose	0.11157182	0.039083369	-79	4
	Maltose	0.14874004	0.087784777	-32	
	Maltotriose	0.02603717	0.013766482	76	
PC1	Glucose	0.04139276		-90	25
	Maltose	0.05287774		21	
	Maltotriose	0.01401116		76	
PC2	Glucose	0.03449744		-58	26
	Maltose	0.04826322		28	
	Maltotriose	0.02665399		54	
NC	Glucose	0.02183936	Reference		0
	Maltose	0.06669746			
	Maltotriose	0.05745075			
Amylase	Glucose	0.03121545			
	Maltose	0.03433898			

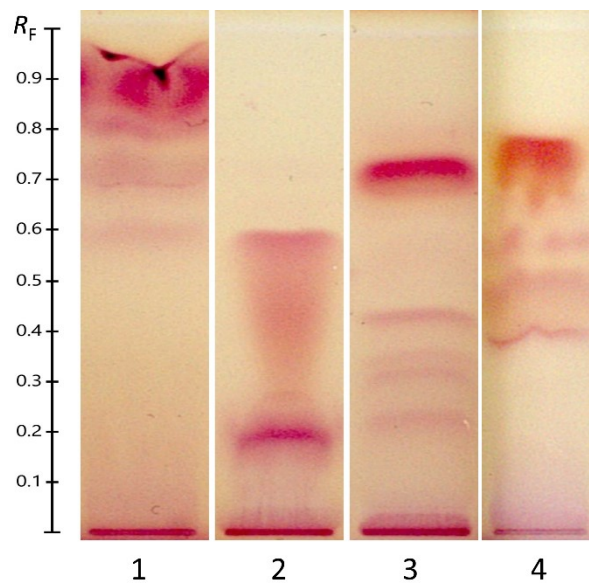


Fig. S1 HPTLC-Vis chromatograms for mobile phase optimization with different ratios of 2-butanol/ammonia (25%)/pyridine/water: **(1)** 10:5:17:13, **(2)** 19:5:8:13, **(3)** 19:5:17:6, and **(4)** 19:5:17:13 (all V/V/V/V); HPTLC silica gel 60 plate derivatized with the ninhydrin reagent and detected at white light illumination (remission and transmission mode).

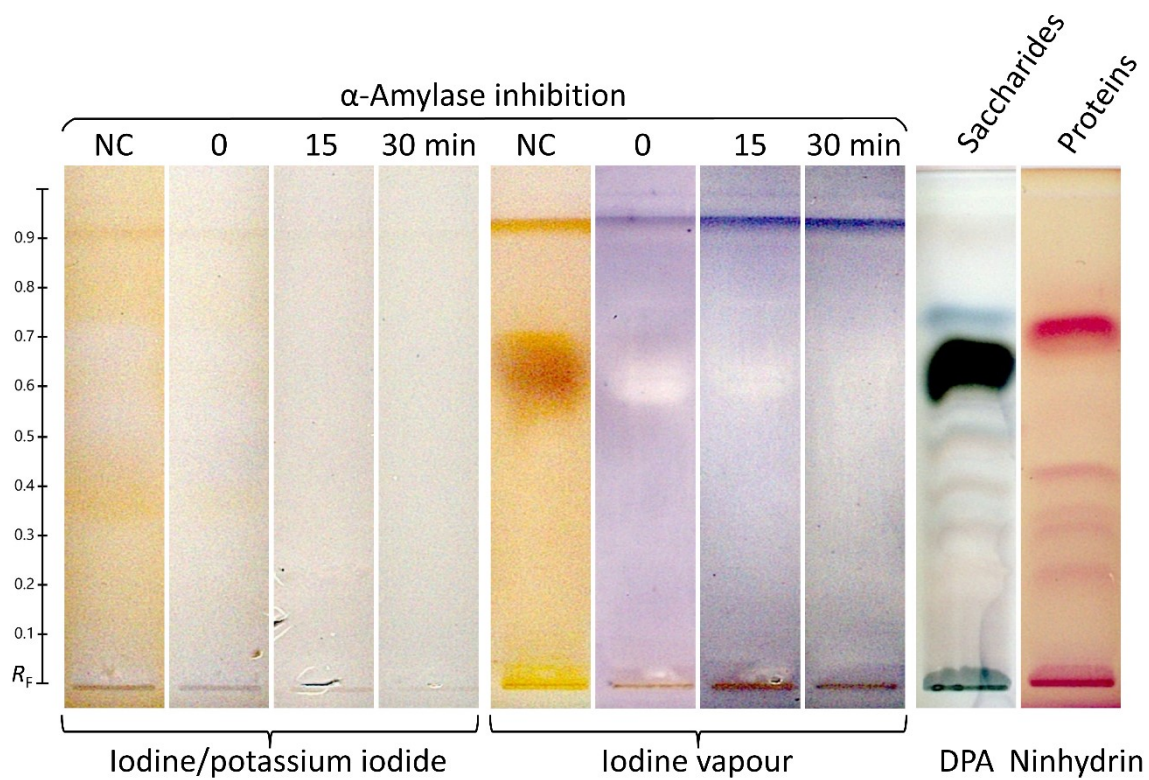


Fig. S2 HPTLC-Vis chromatograms showing separated refined wheat extract (5 μ L/band) with different incubation times (0–30 min) of the amylolysis using either iodine/potassium iodide or iodine vapour as derivatization for detection of α -amylase inhibition in comparison with derivatization with the ninhydrin reagent for proteins and the diphenylamine aniline phosphoric acid reagent (**DPA**) for saccharides. As a negative control (**NC**), the assay was performed without enzyme. HPTLC silica gel 60 plates developed with 2-butanol/ammonia (25%)/pyridine/water 19:5:17:6 (V/V/V/V) up to 65 mm and documented at white light illumination (remission and transmission mode).

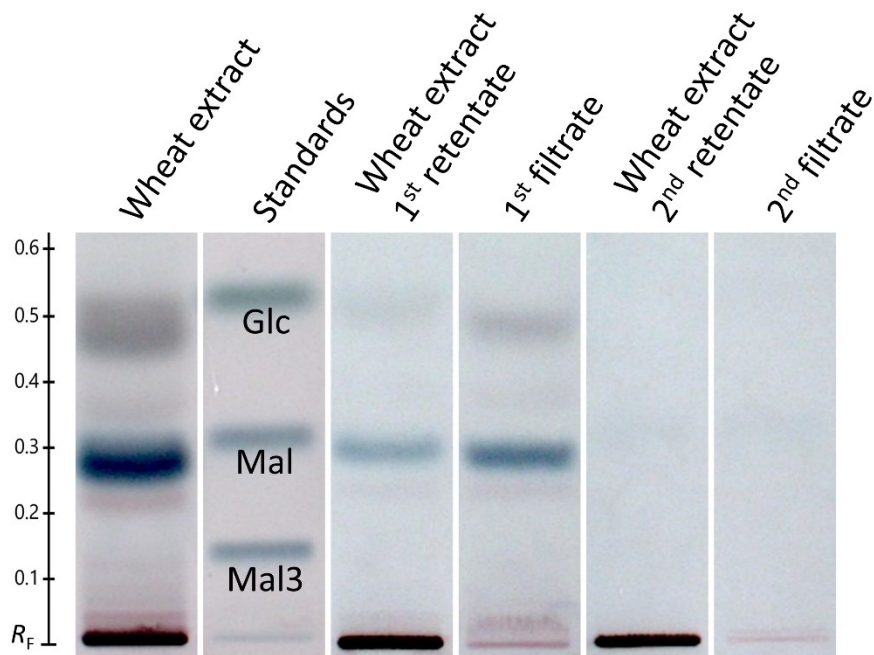


Fig. S3 HPTLC-Vis chromatograms showing saccharide evaluation of the crude and filtered refined wheat extract (5 $\mu\text{L}/\text{band}$) and the corresponding filtrate (10 $\mu\text{L}/\text{band}$). The wheat extract was filtered once or twice for 30 min with a 3k Da Amicon Ultra-0,5 filter. For comparison, reference standards (1 $\mu\text{g}/\text{band}$) glucose (Glc), maltose (Mal) and maltotriose (Mal3) were applied on HPTLC silica gel 60 plate, developed with acetonitrile/water/2-propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the diphenylamine aniline phosphoric acid reagent and documented at white light illumination (remission and transmission mode).

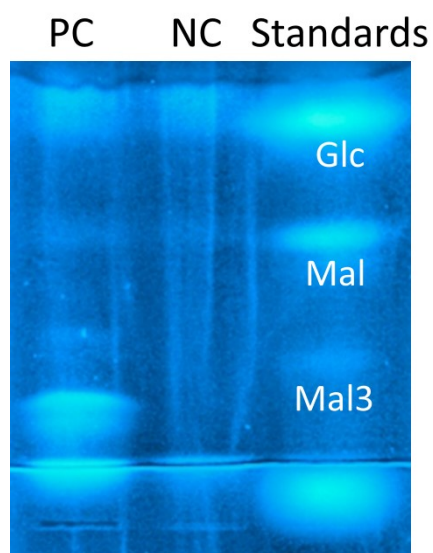


Fig. S4 NP-HPTLC-nanoGIT chromatogram (amylolysis with 45.5 mU/band α -amylase + 2 μ g/band soluble starch) after both on-surface pre- and main incubation of the positive control acarbose (**PC**, 5 μ g/band) and the negative control bi-distilled water (**NC**). For comparison, reference standards (1 μ g/band) of glucose (Glc), maltose (Mal) and maltotriose (Mal3) were applied on HPTLC silica gel 60 plates, developed with acetonitrile/water/2 propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the *p*-aminobenzoic acid reagent and documented at FLD 366 nm.

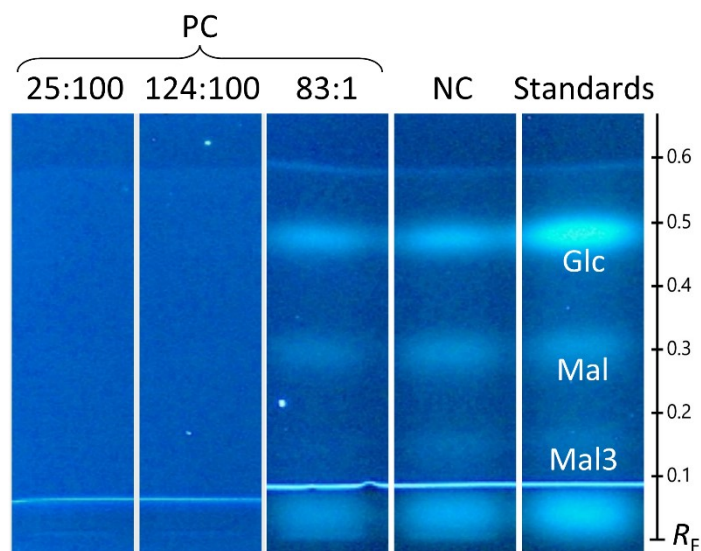


Fig. S5 Evaluation of an appropriate acarbose concentration as the positive control (**PC**) with an in-vial pre-incubation (30 min) and on-surface incubation (60 min). The pre-incubated enzyme-inhibitor mixture (11 $\mu\text{L}/\text{band}$) contained either acarbose (0.4 $\mu\text{g}/\mu\text{L}$) + α -amylase (4.5 $\text{mU}/\mu\text{L}$) in 25.1 μL (**25:100**), acarbose (80 $\text{ng}/\mu\text{L}$) + α -amylase (4.5 $\text{mU}/\mu\text{L}$) in 25.1 μL (**124:100**) or acarbose (2.7 $\text{ng}/\mu\text{L}$) + α -amylase (10.3 $\text{mU}/\mu\text{L}$) in 11 μL (**83:1**). The negative control (**NC**) was α -amylase (113.8 mU/band) + bi-distilled water. All samples were overspotted with soluble starch (2 $\mu\text{g}/\text{band}$). For comparison, reference standards of glucose (Glc) 0.5 $\mu\text{g}/\text{band}$, maltose (Mal) and maltotriose (Mal3) each 1 $\mu\text{g}/\text{band}$ were applied on HPTLC silica gel 60 plates developed with acetonitrile/water/2-propanol/acetone 6:1.5:2:0.5 (V/V/V/V) up to 70 mm, derivatized with the *p*-aminobenzoic acid reagent and documented at FLD 366 nm.

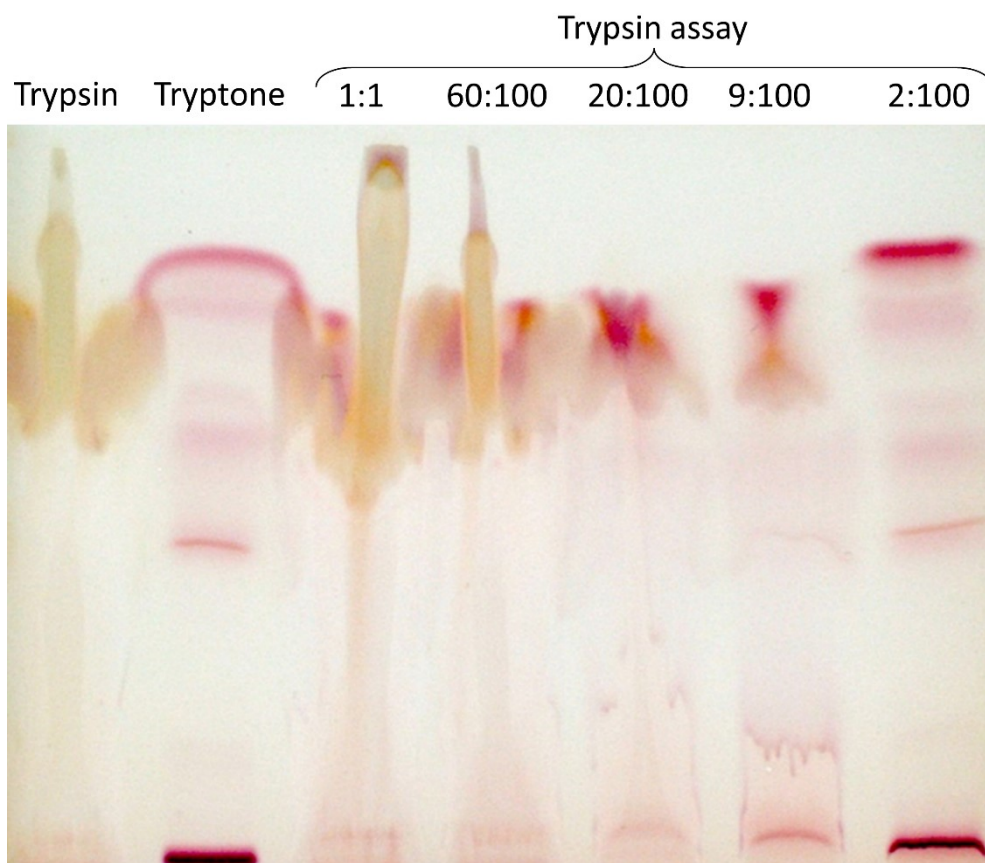


Fig. S6 NP-HPTLC-nanoGIT (proteolysis)-Vis chromatogram of different ratios of trypsin to tryptone (**1:1–2:100**), trypsin (3 µg/band) and tryptone (5 µg/band). For the trypsin assay, tryptone (5 µg/band) was overspotted with different volumes (0.4–18.5 µL) of 0.27 µg/µL trypsin. The HPTLC silica gel 60 plate was developed with 2-butanol/ammonia (25%)/pyridine/water 19:5:17:13 (V/V/V/V) up to 70 mm, derivatized with the ninhydrin reagent and documented at white light illumination (remission and transmission mode).

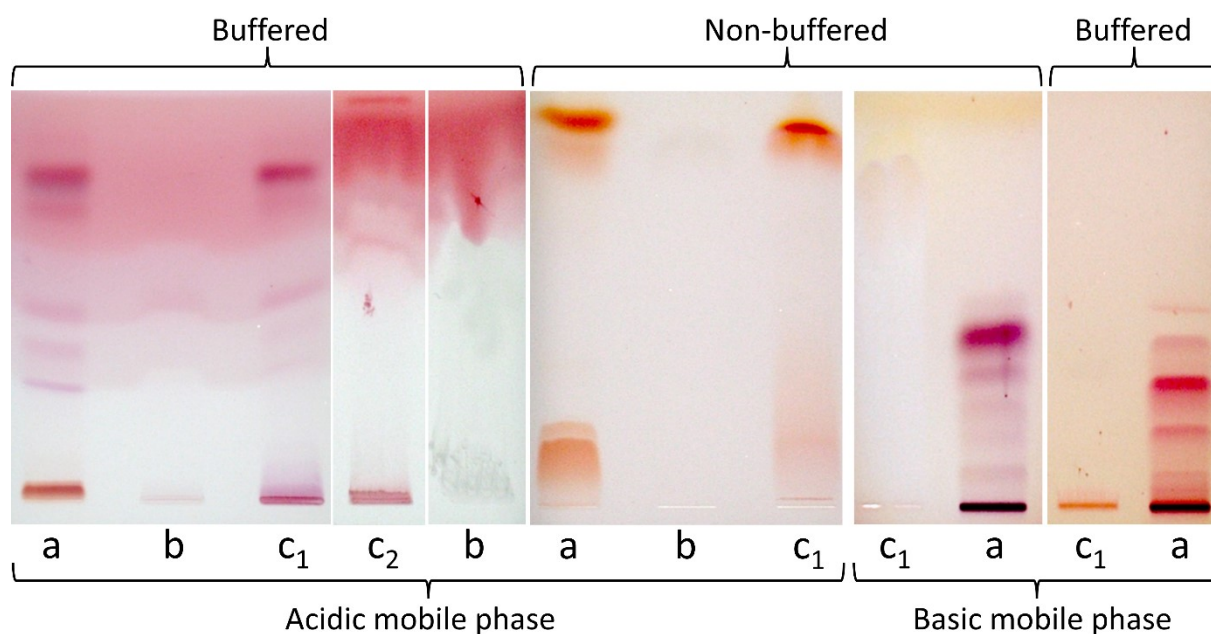


Fig. S7 RP-HPTLC-nanoGIT (proteolysis)-Vis chromatogram of the influence on the peptide separation and derivatization of an acidic or basic mobile phase on HPTLC RP-18 W plates (non-)buffered with citrate-phosphate buffer (pH 12). The trypsin assay (5 μ L/band, tryptone overspotted with trypsin) were applied with a trypsin/tryptone ratio of 1:20 (**c**₁) or 1:100 (**c**₂). Tryptone (**a**, 5 μ g/band) and trypsin (**b**, 0.25 μ g/band) were applied beside. The plates were developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) or 2-propanol/ammonia (25%)/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the ninhydrin reagent and documented at white light illumination (remission and transmission mode).

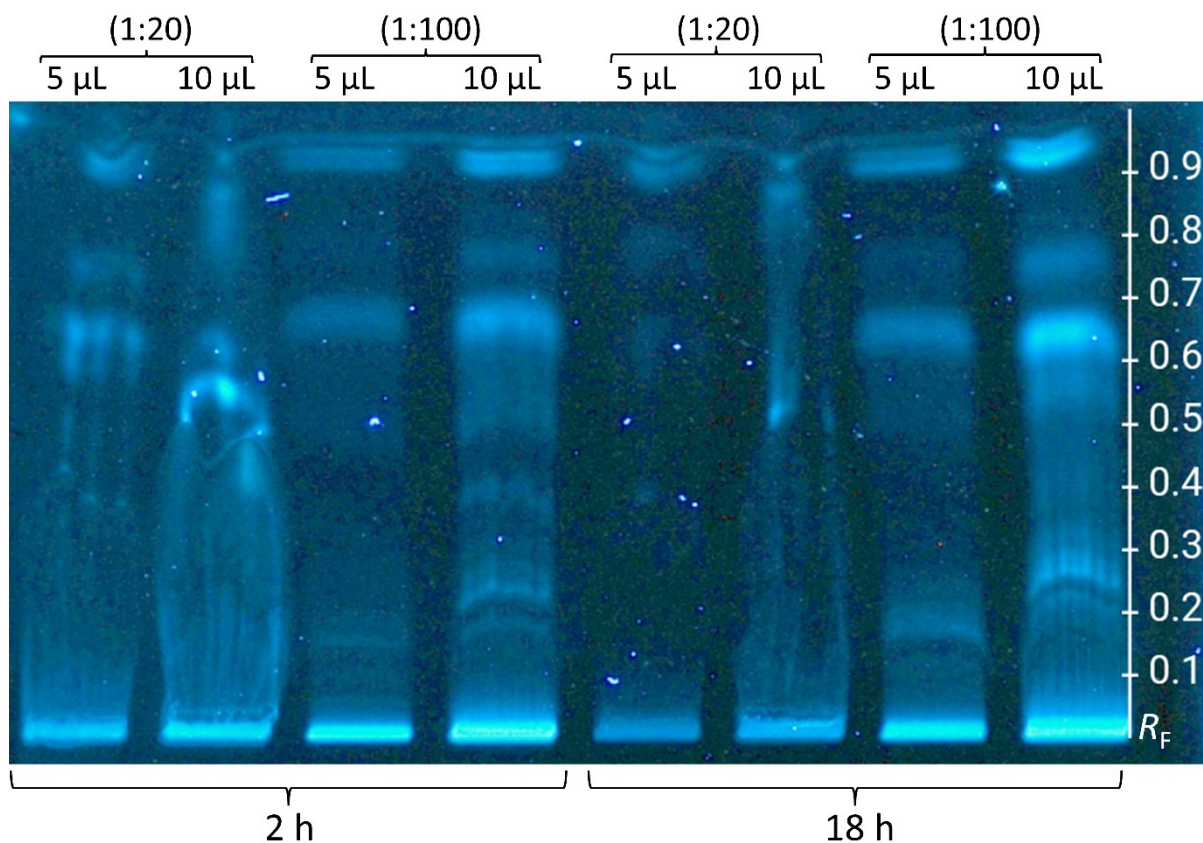


Fig. S8 RP-HPTLC-nanoGIT (proteolysis)-FLD chromatogram showing the influence of the enzyme-substrate ratio (**1:20** or **1:100**) and incubation time (**2** or **18 h**) on the in-vial trypsin-casein assay and the application volume (**5** or **10 µL**) on the peptide separation on HPTLC RP-18 W plates. The in-vial trypsin-casein assay was 100 µL casein (1 µg/µL) with trypsin (0.33 µg/µL) in the ratio of 1:20 or 1:100. The plate was developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the fluorescamine reagent and documented at FLD 366 nm (enhanced mode).

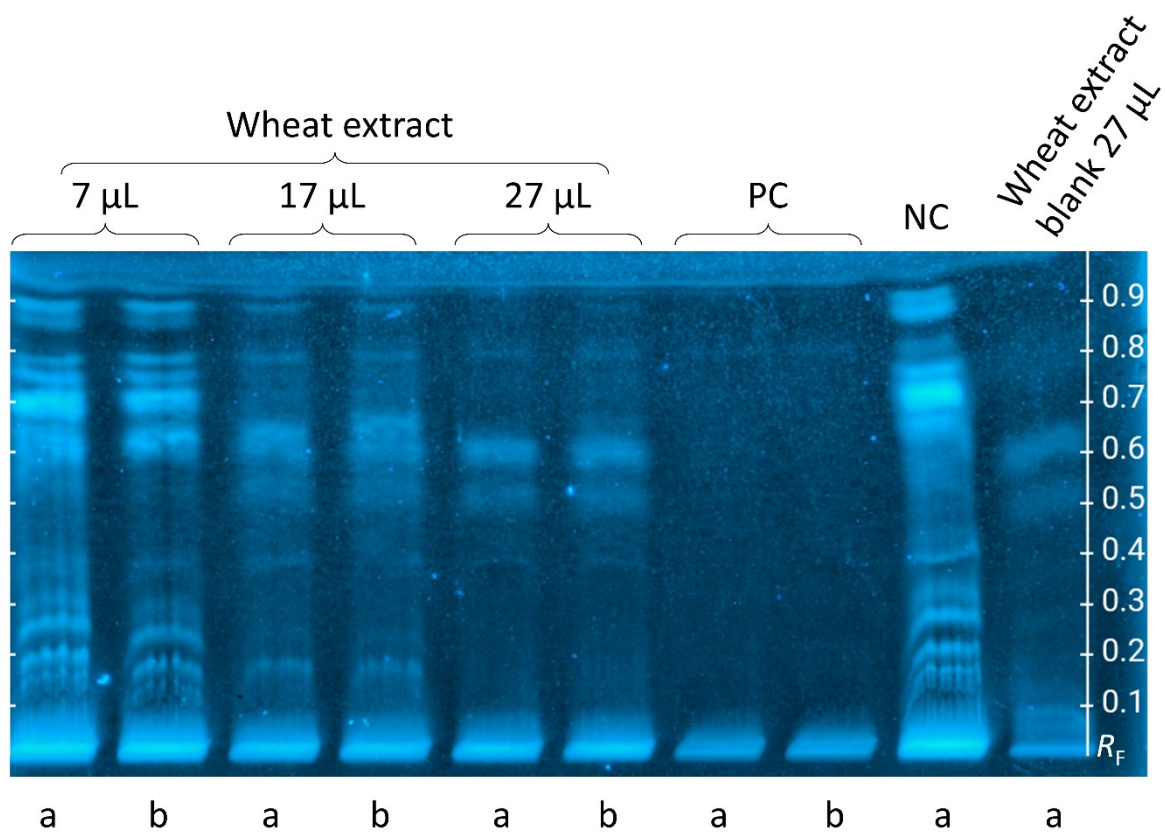


Fig. S9 RP-HPTLC-nanoGIT (proteolysis)-FLD chromatogram of the influence of pre-incubation times of 15 min (**a**) or 30 min (**b**) with different volumes of wheat extract (7, 17 and 27 μL) or a trypsin inhibitor as positive control (**PC**) on the in-vial trypsin inhibition assay. Application of 10 μL /band of each wheat assay, the PC and the negative control (**NC**, trypsin-casein assay (1:100) and a wheat extract blank (27 μL) on HPTLC RP-18 W plates. The plate was developed with 2-propanol/formic acid/acetonitrile 10:1:5 (V/V/V) up to 65 mm, derivatized with the fluorescamine reagent and documented at FLD 366 nm (enhanced mode).