Spatial Distribution of Endogenous Tissue Protease Activity in Gastric Carcinoma Mapped by MALDI Mass Spectrometry Imaging

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In Brief
This sample preparation workflow enables the spatially-resolved monitoring of tissue protease activity in frozen tissue slices using MALDI MS Imaging. It measures the decrease of a general tracer substrate, substance P, and the increase in digestion products in a time-dependent manner. Attenuation of tracer degradation by protease inhibitor mix is concentration-dependent. In a mouse model, we visualized high protease activity in gastric tumor compared to surrounding tissue, and differential protease expression in these tissues was confirmed using quantitative bottom-up proteomics.

Highlights
• New MALDI MS imaging sample preparation workflow reveals tissue protease activity.
• Differential time- and inhibitor concentration-dependence confirm active proteases.
• Mouse gastric tumor displays high protease activity compared to surrounding tissue.
• Proteomic data and biochemical protease activity assay support MALDI MSI results.
Aberrant protease activity has been implicated in the etiology of various prevalent diseases including neurodegeneration and cancer, in particular metastasis. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) has recently been established as a key technology for bioanalysis of multiple biomolecular classes such as proteins, lipids, and glycans. However, it has not yet been systematically explored for investigation of a tissue’s endogenous protease activity. In this study, we demonstrate that different tissues, spray-coated with substance P as a tracer, digest this peptide with different time-course profiles. Furthermore, we reveal that distinct cleavage products originating from substance P are generated transiently and that proteolysis can be attenuated by protease inhibitors in a concentration-dependent manner. To show the translational potential of the method, we analyzed protease activity of gastric carcinoma in mice. Our MSI and quantitative proteomics results reveal differential distribution of protease activity – with strongest activity being observed in mouse tumor tissue, suggesting the general applicability of the workflow in animal pharmacology and clinical studies. 

Proteases control cell and tissue protein homeostasis (1). They influence cell proliferation, tissue morphogenesis and remodeling, and they are therefore associated with many pathological conditions including cancer. Cellular and secreted proteases play important roles during tumor initiation, growth, as well as metastasis, and their activity is not restricted to tumor cells, but affects tumor-surrounding tissue in favor of tumor expansion (2, 3). Hence, they are key drug targets (4).

In the past, various optical methods including (near infrared; NIR) fluorogenic and bioluminescence-based substrate- or activity reporter probes have been used to visualize protease activity in vitro, in cultured cells and in vivo (5, 6). Analytical probes typically enable fluorescence quenching, fluorescence resonance energy transfer (FRET)1, and other optical readouts. Whereas several probes have been described for cell studies and in vivo molecular imaging (7), direct visualization and biochemical investigation of protease activity in tissue sections remains an underexplored field of research. In a notable exception, Withana et al. topically applied fluorescence-quenched activity-based probes to fresh-frozen tissue sections and visualized protease activity at cellular resolution (8).

In this study, we utilize a different analytical approach, matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI), for spatially-resolved analysis of endogenous protease activity in fresh-frozen tissue. MALDI-MS imaging is label-free, and it rapidly localizes biomolecules without prior knowledge of their presence. Sample preparation for MALDI-MS imaging is comparatively fast and, hence, it is of increasing interest for clinical pathology (9, 10). MSI is frequently used for analysis of the in situ distribution of proteins (11, 12), lipids (13, 14), drugs (14), as well as peptides (15), and N-glycans (16, 17). A recent study with a neuropeptide, dynorphin, spotted onto rat brain slices suggested that it may be possible to detect the levels of substrate and peptide bioconversion products simultaneously by MSI (18).
Here, we take this approach one step further and use spray-coating techniques for application of a protease substrate tracer, substance P, onto tissue for effective monitoring of endogenous enzyme activity and of the transient emergence of cleavage products over time at high spatial resolution. We utilize fast MALDI-TOF and high resolving power MALDI-Fourier Transform Ion Cyclotron Resonance (FTICR) MSI to visualize for the first time tissue protease activity in mouse gastric carcinoma using MALDI MSI.

**EXPERIMENTAL PROCEDURES**

**Chemicals—** Substance P was purchased from BACHEM (Bubendorf, Switzerland). Protease inhibitor mix (PIM), trichloroacetic acid (TCA), Folin & Ciocalteu’s phenol reagent, casein (from bovine milk), bovine serum albumin (BSA), pancreatin (from porcine pancreas), Tris/(2-carboxyethyl)phosphine (TCEP) and chloroacetamide (CAA) were obtained from Sigma-Aldrich (Steinheim, Germany). Conductive indium tin oxide (ITO)-coated glass slides and peptide calibration standard II were from Bruker Daltonik (Bremen, Germany), MALDI matrix 2,5-dihydroxybenzoic acid (DHB) from Alfa Aesar (Karlsruhe, Germany), and trifluoroacetic acid (TFA) and ethanol were from Merck (Darmstadt, Germany). Acetonitrile (ACN) was obtained from Fisher Scientific (Waltham, MA), acetonitrile and methanol from WWR Chemicals (Fontenay-sous-Bois, France), and Mayer’s Hematoxylin solution from Sigma-Aldrich. Eosin G Solution 0.5%, magnesium sulfate, sodium bicarbonate, hydrochloric acid and L-tyrosine were all purchased from Carl Roth (Karlsruhe, Germany). All solvents were MS grade. Triton-X-100, potassium hydroxypophosphate (K2HPO4) and sodium carbonate (Na2CO3) were from Merck (Darmstadt, Germany), and trifluoroacetic acid (TFA) and ethanol were from Merck (Darmstadt, Germany). Ammonium formate was purchased from VWR (Darmstadt, Germany), ammonium bicarbonate (ABC) from Fluka Analytical (Munich, Germany), Trifluoroacetic acid (TFA) and formic acid (FA) was from Biosolve Chemicals (Dieuze, France). Sequencing grade modified trypsin was obtained from Promega (Madison, WI).

**Tissue Handling—** Porcine organs were obtained from the local slaughterhouse. Kidney, spleen, pancreas, liver and muscle were prepared in blocks from about 2 × 2 cm, and immediately frozen. Transgenic CEA424-SV40 Tag C57BL/6 J mice with gastric carcinoma were described elsewhere (19). Animal studies were conducted in agreement with ethical guidelines of Heidelberg University and approved by government authorities (Az I-17/07). Stomachs of transgenic mice were removed and immediately frozen. All tissues were immediately frozen and stored at −80 °C until further use.

**Sample Preparation for Protease Activity Assessment by MALDI MSI—** For MALDI MSI analysis, frozen tissue was cut into 10 μm slices on a CM1950 cryostat (Leica Biosystems, Nussloch, Germany) at a temperature of −17 °C and thaw-mounted onto ITO-slides. In washing experiments, slices were rinsed with 2 × 1 ml 50 mM ammonium formate (pH 7 and pH 3). The tissue was vacuum-dried for 15 min at 4 °C. To assess proteolytic in situ activity, one “digest” slide and one “no digest” control slide were prepared per tissue type. For assessment of time-dependent endogenous protease activity on porcine tissue (5 time-points x 5 organs x 2 conditions, i.e. “digest” and “no digest” for a total of 50 MSI data sets), 4 × 1 μl protease inhibitor mix (PIM; 1x) consisting of 2 mM 4-(2-aminoethy)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.3 μM aprotinin, 116 μM bestatin hydrochloride, 14 μM E-64, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 μM leupeptin was applied to each tissue. Additionally, 1 × 1 μl ddH2O was pipetted on each tissue as solvent control. For studies of inhibitor concentration-dependent protease activity on porcine tissue, a dilution series (0 ×, 0.1 ×, 0.25 ×, 0.5 ×, 1 ×, 2.5 ×, 10 ×) of PIM was prepared in H2O, and one μl of each dilution was spotted onto tissue at arbitrarily chosen positions (n = 2). Slides were kept on ice at all times. In experiments using mouse tumor samples, an additional slide was coated with PIM, using the SunCollect (pump system, SunChrom, Friedrichsdorf) and the following conditions: 3 × 15 μl/min, Y-distance 25.5 mm, Speed in X = Low 4, Speed in Y = Medium 1. The final amount of PIM on tissue was 0.2 μl/mm². After drying under vacuum for 3 min at room temperature (RT), porcine or mouse tissues on the “digest” slide (Fig. 1) were covered with a coating with 3.5 μmol/mm² substance P. During the spray process, the slide was kept on a cool pad. After spraying, the slide was dried in desiccator for 3 min at RT. Both slides (“digest” and “no digest”) were incubated simultaneously at 37 °C and 95% humidity in the SunDigest device (SunChrom) for 15 min, 30 min, 45 min, 60 min, 120 min or 360 min in time-course experiments and for 60 min in inhibitor-concentration-dependence experiments (porcine tissues). Mouse tissue was incubated for 30 min at 37 °C instead, as protease activity was apparently higher in snap-frozen mouse tissue than in slaughter-house tissue, but otherwise treated as porcine tissue. Homogeneity of the substance P coating on “no digest” slides was evaluated, and experiment with inhomogeneous coatings were rejected. For MALDI-TOF and MALDI-FT-ICR Mass Spectrometry Imaging— MALDI-TOF MSI with DHB matrix was performed as outlined (14). Porcine tissues were analyzed using an ultraflexxtreme MALDI-TOF/ TOF instrument (Bruker Daltonik) in reflector positive mode and a mass range of m/z 500–2500. Tissues were measured at a lateral resolution of 200 μm, summing up 300 shots per pixel. External quadratic calibration using peptide standard II was performed for all MS instruments. Data was acquired and processed using flexControl V3.4 and flexImaging V4.1 software (Bruker Daltonik). For mouse samples, a rapifleX MALDI-TOF instrument (and flexImaging V5.1 (Bruker Daltonik) was used at a lateral resolution of 50 μm (20 μm for the replicate experiment), summing up 400 shots per pixel. After MALDI-TOF data acquisition, the same slides were analyzed using a 7T solariX XR MALDI FT-ICR (Bruker Daltonik). For imaging, the instrument was used in positive ion mode in an m/z range of m/z 100–3000 with a raster width of 20 μm and 15 laser shots/pixel. The following parameters were used: Ion transfer (Funnel 1 150 V, Skimmer 1 15 V, Funnel RF Amplitude 70 Vpp); Octopole (Frequency 5 MHz, RF Amplitude 350 Vpp); Collision Cell (RF Frequency 2 MHz, RF Amplitude 1200 Vpp); Transfer Optics (Time of Flight 1.5 ms, Frequency 4 MHz, FR Amplitude 350 Vpp); Quadrupole (Q1 Mass 350 m/z); Excitation Mode (Sweep Excitation, Sweep Step time 15 μs). Spectra of mouse tumor stomach were recorded using 512k (FID 0.2447 s) data point transient, corresponding to an estimated resolving power of 33,000 at m/z 400 resulting in a manageable data size of

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1 The abbreviations used are: FRET, fluorescence resonance energy transfer; ABC, ammonium bicarbonate; CAN, acetonitrile; AEBSF, 4-(2-aminoethy)benzenesulfonyl fluoride hydrochloride; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; CAA, chloroacetamide; DHB, 2,5-dihydroxybenzoic acid; EDTA, ethylenediaminetetraacetic acid; FA, formic acid; FTICR, fourier-transform ion cyclotron resonance; H&E, hematoxylin & eosin; ITO, indium tin oxide; LQF, label-free quantification; MALDI MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; NIR, near-infrared; PBS, phosphate buffered saline; PCA, principal component analysis; PIM, protease-inhibitor mixture; RT, room temperature; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; TCEP, Tris(2-carboxyethyl) phosphine; TFA, trifluoroacetic acid; TPAA, tissue protease activity assay.
22 GB. The average detected mass resolution at m/z 1347.73 was 23,000. Internal calibration was done using a lock mass of m/z 780.551 [PC(34:2)+Na]+. Data acquisition was performed using fmsControl (Bruker Daltonik); ion images were obtained from fleximaging 4.1. For verification of substance P and its peptide products, CID fragmentation was used. Data analysis was performed using Biotools 3.2 and Sequence Editor 3.2 software (Bruker Daltonik). Data is available via ProteomeXchange with identifier PXD011104.

Processing of Mass Spectra and Statistical Analysis—To extract mean intensities, we manually drew regions-of-interest (ROI) on tissue around spots with inhibitor (+inhibitor) or without inhibitor (-inhibitor) on “digest” and “no digest” control slides, respectively. Mean spectra of those ROIs were exported into mMass (V5.5.0 (20)) for baseline-subtraction and peak picking (S/N > 5). Intensities of the m/z-values of interest were exported into Excel (Microsoft). Nonlinear regression analysis was performed in Prism 5.0 software (Graphpad Software).

For statistical analysis of the mouse tumor tissue, TOF data sets were converted into imzML file format (21) using the fleximaging converter (resampled to 80,000 data points), whereas the centroided FT-ICR data sets were read directly by an in-house conversion tool. Subsequently, the data sets were imported into R 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) (22, 23) using MALDQuant and MALDQuantForeign packages (24). The segmentation was performed using spatially-aware shrunk centroid clustering (25) (r = 2, k = 2, s = 3) as implemented in Cardina (26). The variable r = 2 to distinguish between tumor and nontumor. The segmentation was performed based on the m/z segment of 798.54 ± 5 and 798.54 ± 0.05 for the TOF and FT-ICR data sets, respectively. In that mass range, the lipid PC(34:1)+K+ with m/z 798.54, known to be enriched in gastric tumor in this mouse model, is present (14). No pre-proc-
centrifugation for 5 min at 10,000 g for 30 min at 37 °C. Protein precipitate was separated by incubation for 30 min at 37 °C. The samples were loaded with a constant flow of solvent A at a concentration of 50% ACN. Protein binding to beads was allowed for 18 min off a magnetic rack, followed by 2 min incubation on a magnetic rack to immobilize beads. The supernatant was removed and beads were washed 2× with 200 μl of 100% ethanol and 1× with 180 μl of 100% ACN. Beads were resuspended in 100 μl ddH2O and sonicated for 5 min in a water bath. Finally, sequencing-grade trypsin was added in an enzyme:protein ratio of 1:40 (5 μl of 0.1 μg/μl trypsin in ddH2O), and beads were pushed from the tube walls into the solution to ensure efficient digestion. Upon overnight incubation at 37 °C and 1000 rpm in a table-top thermomixer, samples were acid-
dified to a final concentration of 0.5% FA and quickly vortexed. Pep-
tides were recovered by immobilizing the beads on a magnetic rack and transferring the supernatant to new PCR tubes (28). MS injection-ready samples were stored at −20 °C.

Proteomics Experimental Design and Statistical Rationale—Samples were diluted with Buffer A (0.1% FA in ddH2O) to enable the injection of 1.5 μg in 10 μl volume. Peptides were separated using the Easy NanoLC 1200 fitted with a trapping (Acclaim PepMap C18, 5 μm, 100 Å, 100 μm × 2 cm) and an analytical column (Acclaim PepMap RSLC C18, 2 μm, 100 Å, 75 μm × 50 cm). The outlet of the analytical column was coupled directly to a Q-Exactive HF Orbitrap (Thermo Fisher Scientific) mass spectrometer. Solvent A was ddH2O, 0.1% (v/v) FA and solvent B was 80% ACN in ddH2O, 0.1% (v/v) FA. The samples were loaded with a constant flow of solvent A at a maximum pressure of 800 bar, onto the trapping column. Peptides were eluted via the analytical column at a constant flow of 0.3 μl/min at 55 °C. During elution, the percentage of solvent B was increased linearly from 3% to 8% in 4 min, then from 8% to 10% in 2 min, then from 10% to 32% in a further 68 min, and then to 50% B in 12 min. Finally, the gradient was finished with 7 min at 100% solvent B, followed by 10 min 97% solvent A. Peptides were introduced into the mass spectrometer via a Pico-Tip Emitter 360 μm OD × 20 μm ID; 10 μm tip (New Objective) and a spray voltage of 2kV. The capillary temperature was set at 275 °C. Full scan MS spectra with mass range m/z 350 to 1500 were acquired in the Orbitrap with a resolution of 60,000 FWHM. The filling time was set to a maximum of 32 ms with an automatic gain control target of 3 × 106 ions. The top 20 most abundant ions per full scan were selected for an MS2 acquisition. The
Fig. 1. Workflow for spatially resolved assessment of protease activity with MALDI MSI. One “digest” slide and one “no digest” slide was prepared. For porcine experiments, protease-inhibitor-mix was pipetted onto the tissue, which was then desiccated. Substance P was sprayed onto the “digest” slide before - and on the “no digest” slide after incubation at 37 °C. During sample preparation, slides were kept on ice to prevent uncontrolled digestion.

Dynamic exclusion list was with a maximum retention period of 40 s. Isotopes, unassigned charges, and charges of 1, 5 to 8, and >8 were excluded. For MS² scans, the resolution was set to 15,000 FWHM with an automatic gain control of 1 × 10⁶ ions and maximum fill time of 50 ms.

Raw files were processed using MaxQuant (version 1.5.1.2) (30). For follow-up data analysis, raw file names as deposited in PRIDE can be matched with the ones in the search output folder by means of the supplemental file “Renaming_Scheme_MaxQuant_Erich.xlsx”. The search was performed against the mouse Uniprot database (20180622_U niprot_mus-musculus_canonical_reviewed; 16970 entries) using the Andromeda search engine with the following search criteria: enzyme was set to trypsin with up to 2 missed cleavages. Carbamidomethylation (C) and oxidation (M/acylation (protein N) term) were selected as a fixed and variable modifications, respectively (30, 31). First and second search peptide tolerance were set to 20 and 4.5 ppm, respectively. Raw files were added in an experimental setup to allow match between runs and replicates of the same condition. The match time window was set to 0.3 min with an alignment window of 20 min. Unidentified features were not matched. Protein quantification was performed using the label-free quantification (LFQ) algorithm of MaxQuant. LFQ intensities were calculated using a minimum ratio count of 1, and minimum and average number of neighbors of 3 and 6, respectively. No MS/MS were required for the LFQ comparison. On top, intensity-based absolute quantification (iBAQ) intensities were calculated. Peptide and protein hits were filtered at a false discovery rate of 1%, with a minimal peptide length of 7 amino acids. The reversed sequences of the target database were used as a decoy database. All remaining settings were set as default in MaxQuant. LFQ values were extracted from the protein groups table and log2-transformed for further analysis. All consecutive steps were performed in Microsoft Excel, Perseus (version 1.6.1.3), and the statistical software environment R version 3.4.3 (22, 32). For differential expression analysis, limma statistic was used (33). Data are available via ProteomeXchange with identifier PXD010369 (Supplemental Data in “IdentifiedProteins_Erich.xlsx”). The protease database MEROPS (https://www.ebi.ac.uk/merops/) was used for protease annotation.

RESULTS

Monitoring of Endogenous Tissue Protease Activity Using MALDI MS Imaging—The pathophysiological importance of proteases has been known for a long time and current photonic tracer-based measurements in tissues may have limitations such as quenching, autofluorescence, and photo bleaching. In contrast, MALDI MSI is a label-free technology that can circumvent such limitations and eventually enable spatially resolved monitoring of endogenous protease activity in relevant tissues. To establish systematic analysis for MSI-based visualization of tissue protease activity (Fig. 1), we chose the undecapeptide substance P (RPKPOQFFGLM; [M+H]⁺ = m/z 1347.7354) as a tracer protease substrate that the MEROPS database lists as a substrate of many proteases of different enzyme classes and diverse origins including mammalian metalloproteases, but also aspartic-, cysteine, and serine-proteases (34) (supplemental Table S2).

To this end, we spray-coated various porcine tissues with this substrate prior (“digest” slides) or after (“no digest” slides) incubation for up to six hours at 37 °C (Fig. 2 and 3). No MALDI-TOF MS signals at m/z 1347.7 or the putative substance P cleavage products m/z 900.5 and m/z 1104.6 were observed in average spectra of any porcine tissue (supplemental Fig. S1–S5). Analysis of substance P sprayed onto an ITO slide without tissue revealed no trace of these fragment peptides, confirming that in-source decay was not responsible for their presence in tissue images (supplemental Fig. S31). This is the prerequisite to assure no overlaying endogenous substance P signal. Generated peptides have been observed for specific substrate-tissue-combinations like dynorphin B on rat brain (18). The observed time-dependent and transient generation of such products suggest that substance P is not merely sequestered into the tissue, but truly digested. MSI of “digest” slides of spleen, liver, kidney, and pancreas to which protease inhibitor mix (PIM; four spots each in arbitrary positions) had been applied, revealed a fast and progressive decrease in m/z 1347.7 ion intensity in regions without PIM. Degradation of the protease tracer was markedly attenuated for 60 to 120 min on the PIM spots (Fig. 2 and supplemental Fig. S7 to S9). In contrast, in muscle, a tissue noted for its low protease activity, no substance P degradation (and consequently no attenuation by PIM) was observed (Fig. 2B). Interestingly, differences in cleavage kinetics and in susceptibility to PIM inhibition were observed for putative cleavage products m/z 1104.6 and m/z 753.4 versus...
m/z 900.5. This suggests that the latter product might be generated by distinct slower or less abundant proteases that are less efficiently inhibited by PIM. Subtle differences were noted for tracer cleavages in different tissues (Fig. 2 and supplemental Fig. S7 to S9). Evaluation of average spectra of PIM-spotted-versus inhibitor-free tissue areas confirmed the conclusions drawn from MS images (Fig. 3).

To assess the time-courses of substance P degradation and transient formation of its proteolytic cleavage products for inhibitor-soaked and PIM-free tissue regions, we plotted mean “digest”-to-“no digest” ratios for substance P and TIC-normalized ion intensities of m/z 1104.6, m/z 900.5 and m/z 753.4 for four inhibited and noninhibited regions (Fig. 4 and supplemental Fig. S10). In spleen and liver, PIM-induced attenuation of substance P degradation was highly significant (p < 0.0001). In contrast, in tissues with very fast decomposition of the tracer (pancreas and kidney) the PIM effect was less significant, thus reflecting high variance of the data at time points <1 h.

Detailed analysis of the cleavage products confirmed the notion that m/z 900.5 transiently accumulated in the presence of PIM, whereas m/z 1104.6 and m/z 753.7 did not (Fig. 4 and supplemental Fig. S10). Whereas this finding may suggest that two sets of proteases play prominent roles in the -PIM (1347.7 → 1104.6 → 753.4 → X) and +PIM (1347.7 → 900.5 → X) cases, follow up studies will be required to test whether the method presented here can be refined to ultimately analyze selective protease action.

Having established the transient generation of PIM-dependent cleavage products as an indicator of a true enzyme reaction being monitored (rather than some nonspecific adsorption or sequestration), we wondered whether PIM-induced attenuation of tracer cleavage was concentration-dependent. Therefore, we used different working concentrations of the PIM stock (10 µM, 2.5 µM, 1.0 µM, 0.5 µM, 0.25 µM, and 0.1 µM) to mimic different concentrations of a single compound inhibitor and spotted various dilutions onto test tissues and incubated them for 60 min. Notably, PIM spots on the “digest” slide indicated an inhibitor concentration-dependent attenuation of substance P degradation (Fig. 5 A and supplemental Fig. S11 to S15). Noninhibited tissue showed very low substance P signals in all cases. Consistent with earlier experiments, muscle displayed essentially no protease activity. All other tissues showed a nonlinear increase of normalized substance P ion intensity with increasing protease-inhibitor-concentration.
**Fig. 4.** Transient presence of substance P cleavage products is modulated by protease inhibitor mix. Ion intensities corresponding to substance P (m/z 1347.7) were extracted from mean spectra of the areas with (orange line) and without (blue line) inhibitor mix (S/N >5). “Digest”-to-“no digest” ratios of ion intensities are plotted. In most tissues degradation of substance P was significantly slowed down by presence of protease inhibitor mix, as assessed by two-tailed ANOVA test (*p* < 0.0001 ****; *p* < 0.001 ***; *p* < 0.01 **; *p* < 0.05 *). TIC-normalized ion intensities of putative substance P cleavage products m/z 1104.6, 900.5 and 753.4 that are generated transiently are plotted versus the incubation time. Note that m/z 900.5 recorded higher intensity in protease inhibitor-containing than noncontaining regions.

**Fig. 5.** Increasing dilutions of the inhibitor mix promote a concentration-dependent decrease in substance P ion intensity. Protease inhibitor mix was diluted and pipetted onto each tissue (10 x, 2.5 x, 1.0 x, 0.5 x, 0.25 x, 0.1 x). “Digest” slide and “no digest” slides were prepared and incubated for 60 min (*n* = 2). A, One replicate for liver tissue of a substance P (m/z 1347.7) MALDI-TOF ion image is shown as an example. B, The “no digest”-to-“digest” intensity ratio was plotted for inhibited (filled circles) and noninhibited (empty squares) regions. Nonlinear curve fitting with variable slope and four parameters was used.
centration. We calculated “substance P survival” factors based on the ratio of substance P ion intensities at the highest PIM concentration and in the uninhibited case. Pancreas had the lowest value (~0.4) suggesting that even at the highest PIM concentration digestion of substance P could not be stopped. Spleen (~0.7) ranked as the tissue with the second-highest protease activity, whereas substance P digestion in liver and kidney could be stopped entirely with 10x PIM concentration (ratio ~1) (Fig. 5B).

To independently test this rank order of protease activities, we developed a protease activity assay for tissue extracts based on the method by Cupp-Enyard (supplemental Fig. S17A). Pancreatin, an extract of active enzymes from bovine pancreas, and denatured pancreatin served as controls (supplemental Fig. S17B). Tyrin calibration curves were highly reproducible in absorption values and showed linear regression (supplemental Fig. S17C). Using casein as a protease substrate (because substance P does not contain tyrosine), we quantified tissue protease activity for each porcine tissue. As observed with MALDI MSI, pancreas showed the highest activity of 8.1 ± 0.8 U/g extracted protein. Interestingly, the rank order of protease activities (supplemental Fig. S16): pancreas > spleen > liver > kidney > muscle) was in line with the one derived from MSI (Fig. 5).

Differential Distribution and Localized Inhibition of Gastric Tumor Protease Activity Visualized by MALDI MS Imaging—Next, we sought to translate this new technique for visualization of endogenous protease activity to preclinical studies in tumor-bearing mice. We had used transgenic CEA424-SV40 Tag C57BL/6 J mice that develop spontaneous gastric tumors in situ in earlier preclinical MSI studies of drugs and their pharmacodynamic actions and identified the potassium aduct of phosphatidyl choline PC(34:1) as a lipid tumor marker in these mice (12, 14). To assess spatial distribution of endogenous protease activity and its attenuation by PIM, we resected a gastric carcinoma-bearing stomach from a transgenic CEA424 mouse and a control stomach from a wild type mouse. Apparently, fresh-frozen mouse tissue displayed higher residual protease activity than porcine tissue obtained from a slaughterhouse (supplemental Fig. S19). Hence, we reduced the incubation time to 30 min (instead of 60 min). No signals of endogenous peptides interfered with m/z corresponding to substance P signal or its cleavage products in tumor bearing or wild-type mouse stomach, as no such signals were observed in the absence of substance P coating (supplemental Fig. S20 to S26). Using DHB matrix, MS images were first acquired on a rapifleX MALDI-TOF MS and then on a SolarIX MALDI-FT-ICR MS. To finally confirm the suspected identity of the four tracer peptides analyzed throughout this study (m/z 1347.7, 1104.6, 900.5 and 753.4), we performed FT-ICR MS CID fragmentation of the respective precursors on the mouse tissue and demonstrated their origin from substance P based on b-ion series (supplemental Fig. S18). Following H&E staining of the sections, tumor, mucosa, the tumor-mucosa transition zone, and the muscularis mucosae were annotated (Fig. 6C).

Predictive segmentation of the putative tumorous tissue by spatially-aware shrunken centroid clustering based on small molecule patterns recorded in reflector positive mode mimicked the distribution of the tumor marker lipid PC (34:1) + K⁺ (m/z 798.5) supporting the utility of the algorithm in oncology studies (Fig. 6A/6B). Results obtained with both imaging technologies were qualitatively similar, but spectral as well as image quality were higher with the FT-ICR, owing to its superior resolving power and mass accuracy (Fig. 6A/6B). Data acquisition with the rapifleX MS was 10-times faster. Substance P ion intensity was prominently and selectively reduced (and cleavage products m/z 1104.6 and 753.4 concomitantly increased) in tumorous tissue when compared with surrounding mucosa and muscularis mucosae. Inversely, the peptide RPKPQQF (m/z 900.5) was depleted in the tumor but enriched in mucosa. Consistent with results in porcine tissues, PIM pretreatment attenuated substance P cleavage, markedly reduced generation of two cleavage products but had little effect at best on accumulation of RPKPQQF (Fig. 6A/6B). Pixel-wise statistical analysis of tumorous and non-tumorous tissue (each ± PIM) substantiated the interpretation that protease activity, as monitored by MALDI-MSI, was differentially distributed, more pronounced in tumor tissue and susceptible to inhibition by PIM (Fig. 6D). Tissue protease activity of tumor-bearing- and wild-type stomach was additionally measured at higher spatial resolution (20 μm), and the distribution of peptides was confirmed (supplemental Fig. S27, S28). Protease activity is susceptible to the local tissue environment (e.g. salt-concentration, pH, and co-factors). To assess possible confounding effects of the local environment on protease activity, we washed the tissue prior to application of substance P with ammonium formiate at pH 7 or pH 3. Protease activity was substantially reduced after washes at pH 7 but unaffected at pH 3 (supplemental Fig. S29A, S29B). Washes at both pH effectively converted potassium adducts of PC (34:1) to the corresponding protonated form (supplemental Fig. S29C).

Quantitative Proteomics Analysis Reveals Differential Protease Expression in Tumor and Nontumor Tissue—Next, we wondered if differential proteolytic activity assessed by MSI in tumor and nontumor tissue in mouse stomachs corresponded with differential expression of proteases in these tissues. To this end, tumor-bearing mouse stomachs (n = 3 biological replicates) were dissected into tumor (T) and nontumor (NT) tissues (supplemental Fig. S38 to S40). Whole stomachs from a WT mouse served as a reference. To quantitatively analyze differential protease expression, we performed a bottom-up proteomic experiment with label-free quantification and identified 3394 proteins. Filtering with the gene ontology term “proteolysis” in the mouse and the EC class 3.4.9.- (peptidases) extracted a list of 104 identified proteases (Fig. 7A). Subjecting these proteases with their associated LFQ values
Tissue Protease Activity Mapping by MS Imaging

A MALDI-TOF (RP=10 000; mass accuracy < 30ppm)
- Inhibitor
- Shrunken Centroid Clustering
  m/z 798.5
  Substance P
  m/z 1347.7
  RPKPQQFFGLM
  m/z 1104.6
  RPKPQQFFG
  m/z 900.5
  RPKPQQF
  m/z 753.4
  RPKPQQ

B MALDI-FT-ICR (RP=30 000; mass accuracy < 3ppm)
- Inhibitor
  m/z 798.54
  m/z 1347.73
  m/z 1104.59
  m/z 900.50
  m/z 753.43
  + Inhibitor
  - tumourous (NT)
  - non tumourous (NT)

C

D

log10 intensity a.u.

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in a principal component analysis (PCA) revealed three clearly distinct groups according to their respective tissue origin (Fig. 7B). The same was achieved when applying hierarchical clustering analysis (Fig. 7C). This contrasted with hierarchical clustering analysis performed on all identified proteins, where NT samples grouped either with WT or T samples, despite clear distinction by PCA analysis (supplemental Fig. S41).

Collectively, these results indicate that expression levels of proteases differ between tumor and nontumor tissues, and that their collective expression pattern is a more powerful indicator for tissue origin than the expression profile of the total proteome.

**DISCUSSION**

Despite the development of photonic probes for spatially resolved monitoring of endogenous tissue protease activities, there is a demand for label-free methods that are not subject to autofluorescence, fluorescence quenching and other interferences and that enable visualization of transient intermediary products. Here, we have provided a proof-of-concept at high spatial and high mass resolution to suggest that MALDI-MSI of tissues spray-coated with substrate peptides such as substance P enables monitoring of protease activity in various tissues. It is beyond the scope of this initial study to elucidate the identity of the proteases involved in cleavage of the tracer peptide. Reference to the MEROPS peptidase database suggests that metalloproteases might be involved. Most proteases that potentially have substance P as substrate (supplemental Table S2) and produce the peptides SubP (1–7) m/z 900.5 or SubP (1–9) m/z 1104.6 are expected to generate SubP (1–7), fewer expected to generate SubP (1–6) followed by SubP (1–9). These proteases belong to the group of matrix-metalloproteases (e.g., endopeptidase-24.11 (3.4.24.11), https://enzyme.expasy.org/EC/3.4.24.11) (36).

**Fig. 6.** Gastric tumorous tissue displays more pronounced protease activity than nontumorous tissue. The stomach of a transgenic CEA424-SV40 Tag-positive tumor-bearing mouse was dissected and immediately frozen. Two cryosections were melted onto an ITO slide, half of which was immediately covered with protease inhibitor mix. All tissues were covered with substance P and incubated for 30 min at 37 °C. MALDI MS ion images were acquired with 50 μm spatial resolution on a rapifleX TOF instrument (A) and subsequently on a SolarIX FT-ICR (B). A, B Automated identification of tumorous (T) and nontumorous regions (NT) by segmentation with spatially aware shrunken centroid clustering. Ion images of the tumor marker lipid(6) m/z 798.5 (PC (34:1) + K+), m/z 1347.7 (substance P) and peptide cleavage products (m/z 1104.6, 900.5, 753.4) are shown. Note that areas of highest concentration of m/z 753.4 represent peptide wash-out outside the tissue. MALDI-FT-ICR MS ion images have higher mass accuracy and show the inhibitor mix’ effect on substance P cleavage and the production of truncated peptides. C, After data acquisition slides were H&E-stained, and tumor, mucosa, tumor transition tissue and muscuaris were annotated. D, For pixel-wise statistical analysis, intensities for m/z of substance P and resulting peptides were plotted for of all pixels on a logarithmic scale. Hypothesis testing was performed using two-tailed unpaired t test (p value < 0.0001 ****; p value < 0.001 ***; p value < 0.01 **; p value < 0.05 *). Scale bar is 2 mm.

**Fig. 7.** Analysis of proteases in TCEA-positive and WT tissue reveal different protease expression patterns. A, Proteomic analysis of wild-type mouse stomach (WT), as well as dissected tumor (T), and nontumor (NT) tissue samples from TCEA-positive mice identified 3394 proteins. Among these, 104 proteases were identified out of 537 UniProt database entries for mouse proteins with the gene ontology term “proteolysis” (GO: 0006508) and the EC class 3.4 (EC3.4.-.-) for peptidases. B, Principal-component-analysis (PCA) of the identified proteases with their respective label-free quantified (LFQ) intensities separated the three tissue groups in the principal components 1 and 2 of PCA space that cover to 45.4% of total variance. C, Hierarchical clustering of the same proteases the grouped the samples according to their origin (WT, T and NT).
that should be inhibited by EDTA in the PIM and serine-proteases (supplemental Fig. S30). Perhaps the composition of PIM under-addresses aminopeptidase and metalloproteases by mainly addressing the inhibition of serine- and cysteine-proteases. More refined follow-up studies with substrates tailored to defined proteases and more selective inhibitors will be important to test whether this method could also be used for selective assessment of target protease families, e.g. cathepsins or distinct matrix metalloproteases.

Our present work does reveal some selectivity in substance P cleavage, for instance selective attenuation of m/z 900.5 generation by PIM, or differential effects of washes at pH3 and 7. However, it mainly assesses global protease activity. Given the well-known pitfalls in MALDI MSI, most notably ion suppression, in heterogeneous tissues one might wonder if the apparent heterogeneous distribution of protease activity in mouse gastric cancer was simply a result of locally different salt concentration or pH (37). Two lines of evidence argue against that: First, washes at pH 3 that completely removed the marker lipid PC(34:1)+K+ and presumably equally regulated pH and salt concentration had no effect on the pattern of substance P cleavage (supplemental Fig. S29). Second, it has been known for some time that protease composition of CEA-TAG± mouse stomachs differ between normal stomach tissue and tumor tissue (38). In tumor tissue mRNA levels of Cathepsin B, H and L as well as MMP2 and MMP13 are increased (38). Although mRNA levels reflect neither protein levels nor activity of proteases, as most proteases are secreted as zymogens and activated via highly regulated pathways, our proteomics data confirm differential protease expression in tumor and surrounding tissue (Fig. 7). It is tempting but inadequate to speculate, which of these differentially expressed proteases (if any) (supplemental Table S3, S4) might be primarily responsible for generation of these substance P cleavage products.

Because it is known from various proteomics studies that tissues are subject to proteolytic degradation not only when incubated at 37 °C, but also when snap-frozen (especially in liver and pancreas) (39), we wondered if our results might be affected by proteolysis. Although this cannot be ruled out entirely, it seems unlikely that such proteolysis would selectively cleave proteases involved in substance P cleavage in the part of the tissue slice but not in others, thus leading to artifactual differences in the distribution of substance P cleavage products. In fact, improved sample-processing using heat-stabilization has been suggested for MALDI MSI to reduce post-mortem degradation (40). In that study, a few peptides were identified that were only visible in the absence of heat stabilization, whereas the majority of peptides reported were unaffected. Again, it is unlikely that peptides unrelated to substance P but originating from tissue autolysis would have the same m/z as substance P cleavage products and thus alter MALDI MS images. In fact, we found no evidence for interfering signals produced by aging-related degradation in digested tissue that was not spray-covered with substance P (supplemental Fig. S32 to S36).

For current clinical diagnosis, often three to five tiny needle biopsy are taken. The method described here could visualize protease activity with high spatial resolution, also on small samples. Homogenizing a biopsy and analyzing for mRNA or protein levels invariably neglects tissue heterogeneity and does not assess tumor-specific mRNA or protein amounts per tissue. Therefore, spatially resolved methods have a clear advantage. With high spatial resolution, now possible with the latest MSI instruments (≈5 μm), also small local differences could be found.

DATA AVAILABILITY

The proteomics and MALDI MSI data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifiers PXD013069 and PXD011104, respectively (41, 42). Additionally, data acquired by MALDI-FT-ICR have been deposited on METASPACE (http://metaspace2020.eu) (43) accessible by the following URLs:

Wild-type without protease inhibitor mix: http://metaspace2020.eu/#/annotations?mdtype=Imaging%20MS&ds=2018-07-21_11h11m58s, Wild-type with protease inhibitor mix: http://metaspace2020.eu/#/annotations?mdtype=Imaging%20MS&ds=2018-07-21_11h20m14s, TCEA positive without protease inhibitor mix: http://metaspace2020.eu/#/annotations?mdtype=Imaging%20MS&ds=2018-07-21_11h23m10s, TCEA positive with protease inhibitor mix: http://metaspace2020.eu/#/annotations?mdtype=Imaging%20MS&ds=2018-07-21_11h24m16s.

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