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Detection of protease activities with the mass-spectrometry-assisted enzyme-screening (MES) system

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Abstract The MALDI-MES provides a rapid, sensitive and reproducible alternative approach to existing analytical techniques for the detection of enzymatic activities that does not require a chromophore or radiolabeling. An improved method is presented, by which enzymes with defined substrate specificities can be detected with a MALDI mass spectrometer in complex protein fractions. In order to demonstrate the utility of the new method, in this study we describe the use of MALDI-MES to detect proteolytic activities in a protein extract from porcine renal tissue, which contained several thousand proteins as visualized by 2D electrophoresis. The analytical procedure is based on covalent immobilization of proteins to beads. By immobilizing proteins, autolytic and proteolytic degradation is prevented and the removal of those molecules from the protein fraction is achieved, which otherwise would interfere with the mass spectrometric detection of the enzymatic reaction products. The enzymatic activity is determined by incubating the immobilized proteins with a reaction-specific probe, followed by the analysis of the reaction mixture with the MALDI-MS after defined incubation times. The presence of the target enzyme is validated by locating a signal, which fits the molecular mass of the expected reaction product in the mass spectrum. To demonstrate how to detect proteolytic activities in this system, the reactions catalyzed by endopeptidase, angiotensin-converting enzyme, kallikrein, renin, and urotensin-converting enzyme were monitored. The experiments showed that the MALDI-MES method is sufficient according the quantification to investigate the effects of inhibitors. This is demonstrated using a specific renin inhibitor to inhibit an angiotensin-I generating enzyme activity in a renal protein extract.

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Abbreviations *MALDI-MS* matrix-assisted mass spectrometry · *MES* mass-spectrometry-assisted enzyme-screening system

Introduction

Proteases play a key role in biological processes such as protein turnover, differentiation, or intracellular and extracellular signaling. About 1.6% of the gene products out of the human genome encode proteases. More than 400 human proteases documented in secondary databases can be delineated in genomic sequence. The number of human proteases reported to be under investigation as drug targets represents approximately 14% of documented proteases [1]. From many of these proteases the endogenous substrates and hence their physiological roles are unknown. In order to identify substrates for orphan enzymes, either surrogate substrates, which can be cleaved by the enzyme in vitro, or the endogenous substrates, which are hydrolyzed in vivo, are used. To determine surrogate substrates, phage display screening [2, 3] or combinatorial peptide library screening are utilized [4]. If cleavage sites are identified, the number of candidate substrates might be narrowed down in silico. However, these cleavage site sequences cannot be searched with any precision, and screening orphan or novel enzymes without further information about the substrate targets is rather speculative. Physiological substrate identification for an orphan protease is much more difficult for the following reasons: i) many proteases show broad specificities; ii) although substrate specificity can be derived from the co-localization of active protease and substrate in developmental stages or particular compartments, this approach cannot be performed quickly; iii) in addition, activation pathways might be involved, in which a post-translational modification of the protease is necessary for full activity. To identify proteases and the

corresponding substrates, detailed biochemical experiments are mandatory.

The classical approach to identify new proteases and their physiological roles circumvents the problems mentioned above. In this case the existing knowledge about the substrate and its cleavage site is used to search for the enzyme which hydrolyses the substrate. For example, the identification of unknown peptide hormones requests the development of methods to detect and identify the prohormone-processing enzymes. After endothelin and its precursor were identified [5] it was possible to use the authentic substrate, big endothelin, for the detection and purification of the endothelin-converting enzyme (ECE) [6]. The classical strategy to identify new enzymes comprises three steps: 1) an assay system is needed to detect the enzyme of interest; 2) a purification procedure must be developed to purify the enzyme to near homogeneity; and 3) the sequence of the purified enzyme must be elucidated.

Proteolytic activities in complex fractions are often detected with spectroscopic methods. With these methods substrates are required that are modified by chromogenic or fluorogenic agents. Such substrates have to be synthesized chemically and have also been demonstrated to alter enzyme kinetics [7]. Radioactive substrates are often preferred because they are chemically identical with the natural substrates and can be detected with high sensitivity. However, most radioactive substrates must be prepared by laborious chemical syntheses. In addition, preparation of radioactive phosphorylated substrates yields a small amount of substrates with a short half-life. Most importantly, measurement of radioactivity is merely a measure of the radioactive isotope and it does not provide any information regarding the identity of the radioactive enzymatic reaction products. Therefore, after the enzymatic reaction, the reaction products must be separated from the substrate before they can be quantified. A further problem with radioisotopes is the hazardous radioactive waste.

Mass spectrometry (MS) is a rapid, sensitive, and accurate quantitative method for analysis of peptides and proteins. Therefore, it is particularly attractive for the analysis of peptidase and protease reactions. MS allows the direct analysis of mixtures of biomolecules without the need for radioactive or chromogenic labeling. An early report about the application of MS in conjunction with liquid chromatography for the analysis of enzyme kinetics was published 1995 by Henion's group [8]. Bothner et al. [9] described the ESI- and MALDI-mass-spectrometry-assisted monitoring of the proteolysis of intact viral capsid proteins, the α-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl-α-glucopyranoside, and the lipoprotein lipase-catalyzed ester hydrolysis of resorufin. Kang et al. [10] demonstrated that relative peak-height ratios of low molecular mass products to substrates determined by MALDI-MS allow the quantitative analysis of enzyme-catalyzed reactions. Recently our group developed a MALDI-MS-assisted method to screen complex protein fractions for defined enzymatic activities [11]. The strategy includes the conversion of proteins into protein libraries by immobilizing the proteins covalently to beads. Enzymatic activities are detected by incubating individual proteins from the library with active-site-directed reaction-specific probes. After defined incubation times, aliquots of the reaction mixtures are analyzed with MALDI-MS. The target enzyme is present if the mass signals from the expected reaction products can be detected.

In this study further improvements in the detection of proteases are demonstrated and new applications are shown.

Experimental

Reagents

All chemicals and enzymes were purchased from Sigma (Deisenhofen) if not stated otherwise. CNBr-activated Sepharose 6 MB beads were obtained from Amersham Biotech (Freiburg). A Bradford assay kit was purchased from Bio-Rad (Munich). The kallikrein substrate (H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Glu-Val-Ser-OH), the tetradecapeptide renin substrate (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH), and the specific renin inhibitor (Z-Arg-Arg-Pro-Phe-His-Statin-Ile-His-Lys (BOC)-OMe were obtained from Bachem (Weil). The urotensin-converting enzyme substrate was synthesized by WITA (Teltow). The MALDI matrix was obtained from (Bruker-Daltronics, Bremen). Deionized water was prepared with a Millipore system (Eschborn). 2DE gel solutions and carrier ampholytes (Witalytes 2–11) were from WITA (Teltow). Porcine kidneys were obtained from the local slaughterhouse.

Instrumentation

2DE was performed with equipment from WITA (Teltow). Silver-stained 2DE gels were scanned using ScanJet 6300C equipment (Hewlett Packard, Palo Alto) at 1,200 dpi. Raw 2DE images were analyzed using the Melanie III software (GeneBio, Geneva).

For pipetting the reaction solutions to the MALDI target, a pipetting roboter (Multiprobe II, Perkin–Elmer, Dreieich) was used. All mass spectra were acquired on a Reflex III MALDI mass spectrometer (Bruker-Daltronics, Bremen). The software package XMASS 5.1 and a 384-microtiter-well format MALDI target, an AnchorChip technology target, were obtained from Bruker-Daltronics (Bremen).

Tissue homogenization and preparation of the protein extract

Porcine kidneys were placed in ice-cooled physiological saline solution immediately after excision and processed within 30 min. The tissue was cut into small pieces (about 1 cm^3), frozen in liquid nitrogen, and stored at –80 °C for 24 h. The frozen tissue was lyophilized and powdered. The freeze-dried powder (1 g dry weight) was suspended for 2 min in 10 mL 20 mM potassium phosphate buffer (pH 7) at 4 °C and homogenized. The homogenate was centrifuged at 30.000 g for 1 h 30 min at 4 °C. The pellet was discarded. One aliquot of the supernatant was used for the immobilization of the proteins. A further aliquot was analyzed by 2DE electrophoresis. Protein concentration was estimated by the Bradford mini-assay method [12].

Gel electrophoresis and image analysis

High-resolution 2DE was performed as described by Klose and Kobalz [13]. IEF was performed in rod gels (inner diameter for the analytical gels 0.09 cm and for the preparative gel 0.15 cm) containing 3.5% acrylamide, 0.3% piperazine diacrylamide, and 4% w/v carrier ampholytes. Renal proteins (50 µg) were loaded per rod

(1.5-mm thickness) and separated according to the WITA protocols [14]. Gels were stained with the MS-compatible silver-staining procedure [15]. Bands were automatically detected without previous contrast enhancement, followed by manual edition if necessary.

Immobilization of the renal proteins

For each experiment, 50 µL from the renal protein extract (protein concentration 14 µg µL^{-1}) and 50 µL water as a control were mixed each with 150 µL 0.1 M $NaHCO_3$, pH 8.3, and 50 µL CNBr-activated Sepharose beads. The mixtures were incubated for 2 h at room temperature on an overhead rotor. After immobilization the beads were blocked with 20 µL 0.2 M glycine in 0.1 M $NaHCO_3$, pH 8.3, for 2 h at room temperature. After blocking, the beads were washed three times with doubly distilled water.

Enzymatic reactions: incubation of the immobilized proteins with protease substrates

Beads (20 µL) containing the immobilized proteins as well as the control beads were transferred each into individual wells of the 384-well microtiter plate. A 20-µL aliquot of a suspension containing the reaction-specific probe (10 µM of peptide) was added. From the reaction mixture 1-µL aliquots were removed in triplicate after 2, 10, 30, and 240 min for mass spectrometric analysis. All incubation experiments were performed at room temperature. For the detection of renin-like activity the reaction-specific probe for renin, tetradecapeptide renin substrate (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH), was used. The incubation of immobilized renal proteins with renin substrate was repeated in the presence of 10 µM of a specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Statin-Ile-His-Lys-(BOC)-OMe.

The reaction-specific probe for the urotensin-converting-enzyme-like activity was Arg-Ile-Lys-Lys-Pro-Tyr-Lys-Lys-Arg-Gly-Pro-Pro-Ser-Glu-Cys-Phe-Trp-Lys-Tyr-Cys-Val, which contains an intramolecular cystine-bridged loop. The final concentration of the peptide in each well was 10 µM. The final volume of each well was 20 µL (including the volume of the beads). A 1-µL aliquot of the reaction mixture was removed after 10, 30, 60, and 120 min for mass spectrometric analysis in triplicates (n=3).

Analysis of the proteolytic reaction products by MALDI-MS

Aliquots from the reaction mixture and from the controls were examined by MALDI-MS. For sample preparation of the reaction mixtures, 1 µL of the reaction mixture was applied onto a 384-format AnchorChip target in triplicate. Next, 1 µL of matrix solution (1:10 dilution of a saturated solution of α-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetonitrile and water containing 0.1% TFA; to the matrix mixture the peptide saralasin (10 nM) was added as an internal standard) was transferred on the dry sample. The mixture was gently dried on the target before introduction into the mass spectrometer. All mass spectra were acquired on a reflectron-type time-of-flight (RETOF) mass spectrometer. Positively charged ions were analyzed in the reflector mode using delayed ion extraction. Spectra were recorded with a 2-GHz data sampling rate. Instrument high voltages were left on between analyses to ensure a stable instrument performance. Unless otherwise stated, the extraction delay time was 150 ns and deflection was used to suppress ions up to m/z 800. In this study, a nitrogen laser with an emission wavelength of 337 nm and 3-ns pulse duration was used. Typically, the laser beam was focused to a 50-mm diameter at an angle of 45° to the surface of the target. Microscopic sample observation was possible. For each sample, 100 single-shot spectra were accumulated, which result from 5 different spots per sample (20 spectra per spot). The complete MALDI-MS analysis was performed automatically using the Reflex III software. All further processing was performed in batch mode using the software package XMASS 5.1. Automated peak picking was performed using the SNAP algorithm provided by XMASS 5.1. This algorithm uses the data points for all recorded monoisotopic mass signals of a peptide to assign an m/z value to the first monoisotopic peak.

Results and discussion

The aim of this study was to demonstrate the utility of the MES method for the detection of proteolytic activities in complex protein fractions. A protein extract from porcine renal tissue was chosen as a complex protein fraction. Analysis of this fraction with 2DE electrophoresis yielded a 2DE gel with several thousand protein spots (data not shown).

With the MALDI-MES method the presence of enzymes with defined catalytic properties in complex protein fractions is detectable. In comparison to chromogenic, fluorogenic, or radioisotope-based assays the method presented here utilizes MALDI mass spectrometry to detect and quantify the reaction products.

The mass spectrometric data give more confidence about the identity of the reaction products than any of the other enzyme assays and therefore avoid false positive results. If the identity of a reaction product is doubtful, mass spectrometry even allows confirmation of the identity, not only by yielding the molecular mass, but also by structural data derived from MS/MS or MALDI-PSD experi-

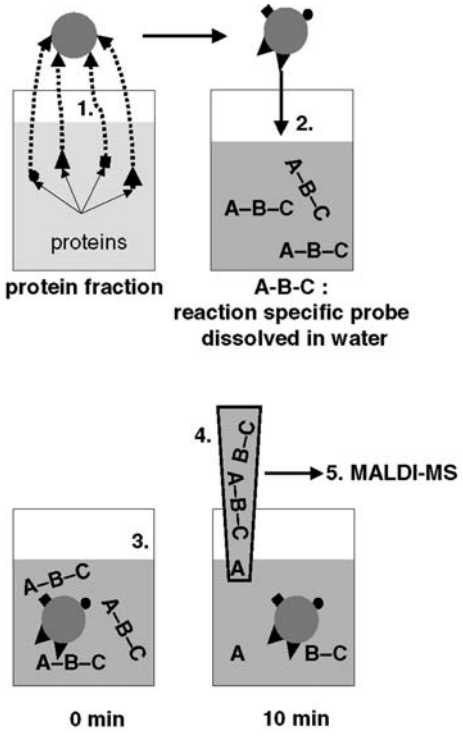

Fig. 1 MALDI-MES steps necessary for the detection of a defined proteolytic activity in a complex protein fraction. *1* Immobilization of the proteins to activated affinity beads. *2* Transfer of the washed immobilized proteins into water, in which the reaction-specific probe is dissolved. *3* The incubation starts as soon as the immobilized beads are mixed with reaction-specific probe. *4* After defined incubation times aliquots are removed for the MALDI-MS analysis of the reaction products

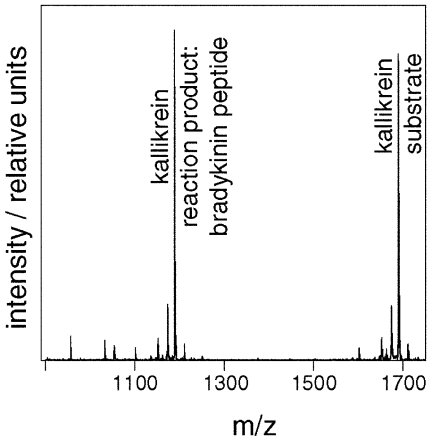

Fig. 2 MALDI-mass spectrum of the reaction mixture of the incubation of kallikrein substrate (1,688.9 Da) with immobilized kallikrein after 1 h of incubation time. The sequence of reaction product of kallikrein, a bradykinin peptide, is H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (1,187.7 Da)

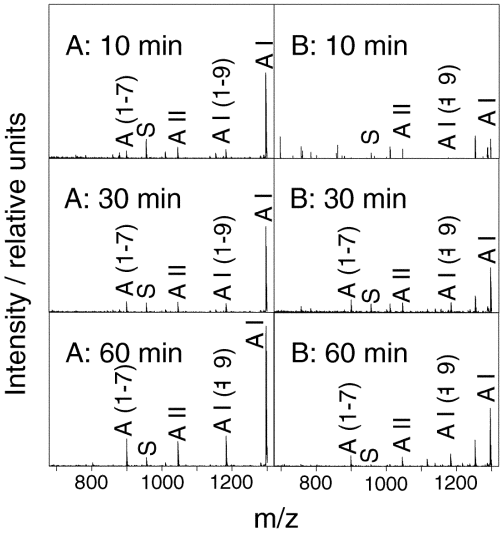

Fig. 3A,B MALDI-MS spectra of the reaction products of the incubation of immobilized renal proteins with renin substrate after different incubation times (10, 30, and 60 min) in the absence (**A**) and in the presence (**B**) of the renin inhibitor. Because the deflection was used to suppress ions smaller than 900 Da and larger than 1,350 Da, the signal of renin substrate is not present. Reaction product of renin-like activity: A I, angiotensin-I (1,295.7 Da); reaction product of the ACE-like activity: A II, angiotensin-II (1,045.5 Da); reaction product of ACE-II-like or other carboxypeptidase activity: A I (1–9), angiotensin-I (1–9) (1,182.6 Da); reaction product of the endopeptidase: A (1–7), angiotensin-(1–7) (898.5 Da). S: internal standard: saralasin (912.1 Da)

ments. To determine enzymatic activities with a mass spectrometer, the enzymatic reaction conditions must fit the requirements for mass spectrometric analysis. These requirements are taken into account by the analytical procedure described below.

General analytical scheme

The analytical procedure comprises five steps and is outlined in Fig. 1. First, the proteins, present in complex mixtures of protein fractions, are covalently immobilized to activate affinity beads. This step is very important for the complete method as it offers several advantages. The immobilization ensures that no biomolecules, which are present in the fraction, will interfere in the mass spectrometric analysis with the reaction products of the enzymatic reaction. In the mass spectra obtained from the reaction mixture containing the immobilized proteins and the reaction-specific probe, only the mass signals of the substrate and the reaction products are present. For example, incubation of immobilized kallikrein with the reaction-specific probe, kallikrein substrate, yielded a bradykinin peptide (Fig. 2). Incubation of the immobilized protein extract from renal tissue resulted in the reaction products angiotensin-I, angiotensin-I (1–9), angiotensin-II, and angiotensin-(1–7) (Fig. 3), suggesting the presence of renin, angiotensin-converting enzyme-II, angiotensin-converting enzyme-I, and an endopeptidase in the complex protein fraction. Renin catalyzes the hydrolysis of renin substrate, an angiotensinogen fragment, to angiotensin-I [16]. Angiotensin-converting enzyme-II was identified recently [17] and was shown to cleave angiotensin-I yielding angiotensin-I (1–9). Angiotensin-converting enzyme generates angiotensin-II from angiotensin-I [18]. Endopeptidase converts angiotensin-I to angiotensin-(1–7) [19]. All four enzymes were reported to be present in renal tissue [20, 21, 22, 23]. The immobilization of the individual proteins in complex fractions is also useful as the proteins cannot degrade each other, thus preventing an inactivation of the enzymes of interest. Because of the immobilization of the proteins, buffer components and inorganic salts can be completely removed. If these substances are present, the signal intensities of the analytes are significantly decreased in the mass spectra, in general by several orders of magnitude.

Second, the proteins, immobilized to beads, are transferred into the reaction wells, which contain the reaction-specific probe dissolved in HPLC-grade water. In the absence of buffer components and salts, optimal conditions for the MS analysis are guaranteed.

Third, the enzymatic reaction starts as soon as the immobilized proteins are mixed with the reaction-specific probe provided that the target enzyme is present in the fraction.

Fourth, after defined incubation times, aliquots are removed from the reaction mixture. Because the enzyme is immobilized to the beads it is not removed from the well and the enzymatic reaction in the aliquot is stopped as soon as the aliquot is removed.

Fifth, the aliquot is transferred to the MALDI target and analyzed by MALDI-MS. Because only a single transfer of the analyte from the reaction well to the MALDI target is necessary, sample loss during the transfer and handling steps is minimized.

The net result of the immobilization of the proteins is a fast, highly sensitive, and artifact-free method for the mass spectrometric determination of enzymatic activity.

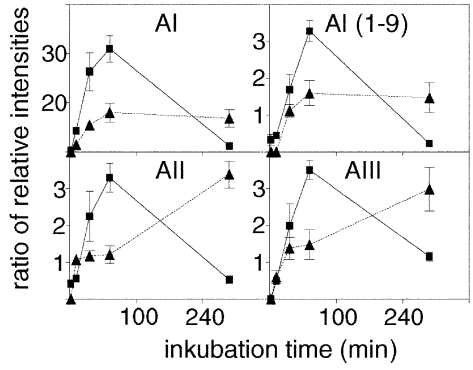

Fig. 4 Incubation-time-dependent amount of reaction products after the incubation of immobilized renal proteins with renin substrate in the absence (*solid line*) and in the presence (*dotted line*) of renin inhibitor. *Abscissa* incubation time. *Ordinate* ratios of the relative MALDI-spectra signal intensities of the reaction products and the relative signal intensities of saralasin (internal standard) (n=3). *AI* angiotensin-I; *AII* angiotensin-II; *AI (1–9)* angiotensin-I (1–9); *A(1–7)* angiotensin-(1–7)

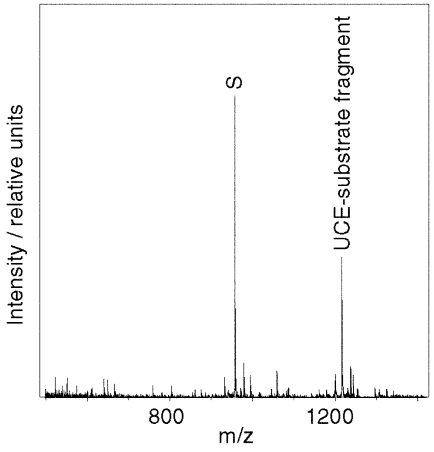

Fig. 5 MALDI-MS spectrum of the reaction product of the incubation of immobilized renal proteins with the urotensin-converting-enzyme (UCE) substrate (2,610.4 Da) after 2 h incubation. Because the deflection was used to suppress ions smaller than 900 Da and larger than 1,350 Da, the signal of UCE substrate is not present. UCE substrate fragment: 1,215.8 Da. S: internal standard: saralasin (912.1 Da)

In this study an improved method compared to the method recently published by our group [11] is described. Main new developments were the following additional steps. First, the signal from the reaction-specific probe (the educt) was excluded because this step significantly enhances the detection limit of the reaction products (Figs. 3 and 5). Second, a peptide was added as an internal standard to the matrix. By using the relative signal intensity ratio of the reaction product to the standard a more precise relative quantification of the reaction product is achieved. This result is in accordance with the study of Kang et al. [10], who investigated and discussed the problem of quantification of analytes with MALDI-MS in detail. Direct quantification by MALDI-MS is difficult because the inhomogeneous distribution of analytes within the sample spot causes poor shot-to-shot and sample-to-sample reproducibility. The inhomogeneous distribution of analytes in a sample depends on many factors, for example, the kind of matrix, the pH, the presence of organic solvents and other additives in the matrix mixture, the complexity and the concentration of the analyte, the presence of contaminants in the analyte, the surface of the target and the drying parameters. Kang et al. [24] obtained very precise standard curves with deuterium-labeled internal standards. Because the synthesis of deuterium-labeled peptides is expensive, we used internal standards, which have molecular masses close to those of the products of the expected proteolytic reactions. As a result we are unable to obtain as precise standard curves as Kang el al. [24], and the quantification has to be termed semi-quantitative. Nevertheless, the precision is sufficient to detect significant differences in enzyme activities as shown in Figs. 3 and 4. For the experiment shown in Figs. 3 and 4, the immobilized protein fraction from a renal extract was used. The immobilized renal protein fraction was incubated in the absence and presence of a specific renin inhibitor with renin substrate. After defined incubation times the reaction products were analyzed by MALDI-MS (Fig. 4). The calculated ratios of the relative signal intensities of the reaction products and the internal standard are shown in Fig. 4. The time-dependent increase of the four reaction products is significantly attenuated in the presence of the renin inhibitor. In the case of angiotensin-I and angiotensin-I (1–9), significantly lower amounts of both peptides were generated within the first 60 min. There was no change in the amounts of angiotensin-I and angiotensin-I (1–9) between 60 min and 240 min in the presence of the renin inhibitor. In contrast, the amounts of angiotensin-II and angiotensin-(1–7) showed a steady increase up to 240 min. In the absence of the renin inhibitor, all curves show a maximum at 60 min. After 240 min, the relative signal intensities of all reaction products were less than 1. This phenomenon may be explained by a nearly complete hydrolysis of all of these peptides. In summary, the curves in Fig. 4 demonstrate that the precision of the semi-quantitative determination of the amount of the reaction products is sufficient to study the effects of inhibitors on enzymatic activities. For the main purpose of MALDI-MES method, the detection of defined enzymatic activities in protein fractions, the precision of the quantification is sufficient. More importantly for this purpose, the identity of the reaction product can unequivocally be determined by mass spectrometry, rendering an erroneous purification of any other than the relevant enzyme unlikely. Furthermore, specific inhibitors can be used, if available, to decide whether a protein fraction contains an enzyme already known. Hence, MALDI-MES appears to be a time-saving method when unknown enzymes with predefined catalytic properties are being purified.

Figure 5 shows a further application for the MALDI-MES method. A peptide fragment of porcine pro-urotensin was synthesized, which contains the cleavage site for an urotensin-converting enzyme (UCE). Urotensin-II is a vasoactive peptide, which was discovered in human tissue re-

cently [25]. In Fig. 5 the expected cleavage product, which results from the hydrolysis of an UCE-like activity, can be recognized, demonstrating that UCE may be present in renal tissue.

Conclusion

The MALDI-MES method has been developed to detect proteases and other enzymes with defined substrate specificities. The mass spectrometric approach enables the sensitive and reliable detection of enzymatic activities even in complex protein mixtures and therefore is a suitable tool for the purification of unknown proteases from body fluids, cells, or tissues. The precision of the semi-quantitative method is sufficient to study the effect of inhibitors to enzymatic activities of complex protein fractions and therefore allows a first characterization of the enzyme of interest.

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