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DETERMINATION OF ENZYME ACTIVITIES BY MASS SPECTROMETRY - BENEFITS AND LIMITATIONS

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ABSTRACT

Enzymatic activities in complex protein fractions are often detected with spectroscopic methods, necessitating substrates, which are modified by chromogenic or fluorogenic agents or with radioactive isotopes. However, both approaches lack the control of the identity of the reaction products risking incorrect positive results. Mass spectrometry-assisted enzyme assays allow the direct and sensitive analysis of the reaction products of the enzymatic conversion of authentic natural substrates and give confidence about the identity of the reaction products. The newly developed mass-spectrometry-assisted enzyme screening (MES) method enables the determination of enzyme activities by mass spectrometry even in raw extracts and cell lysates without sample pre-treatment prior to MS.

INTRODUCTION

In the field of enzyme kinetics spectrophotometric methods are used extensively to monitor product formation during the enzymatic reaction. This most often requires artificial substrates that undergo a change in absorbency at a given wavelength upon turnover. Although this is a simple and effective means for kinetic analysis, the scope of substrates that can be studied is highly restricted.

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Furthermore, natural substrates for the enzyme generally cannot be assayed in this manner. Therefore radioactive substrates are often preferred because of their identical chemical nature to the natural substrates as well as their sensitivity of detection.

However, radiometric assays require the separation of the radioactive products by thin layer chromatography or other chromatographic methods, and subsequent liquid scintillation counting. Optical as well as radiometric methods share the problem, that there is an ambiguity about the fate of the chemical structure of the substrate after the enzymatic conversion. Therefore incorrect positive results cannot be excluded. A different type of detector, which responds universally to all substrates and reaction products, would have definite advantages over spectrophotometric systems. Because enzymatic reactions change the chemical structure of the reactants this change is generally accompanied by a change in the molecular weight. Therefore for the detection of enzymatic activities mass spectrometric techniques are rapid, sensitive and reproducible alternatives. The applicability of mass spectrometry (MS) for the determination of enzyme kinetics has been demonstrated by a number of investigators in the past eight years. The first application of MS in conjunction with liquid chromatography for the real-time analysis of enzyme kinetics was reported in 1995 [1]. Bothner et al. demonstrated that in cases where introduction of a chromophore drastically changes the fate of the reaction as a result of the structural features of the substrate, electrospray MS (ESI-MS) has been found to be especially valuable [2]. Wu et al. reported that ESI-MS could be used as a rapid, sensitive and accurate quantitative assaying tool for inhibitor libraries [3].

Matrix-assisted laser desorption/ionization (MALDI)-MS is generally more robust than ESI-MS towards buffer solutions and is more suitable for complex mixture analysis. It is therefore generally better suited for direct screening of enzyme activities requiring only minimal sample pretreatment. As a typical application MS-based enzyme activity determination was used to elucidate key fluxes in the central metabolism of lysine producing *Corynebacterium glutamicum* during batch culture [4]. Nevertheless a restriction for MALDI-MS for quantitative analysis of small enzymatic reaction products is the interference of matrix signals with analyte signals in the mass range between 22 and 500 Da. An appropriate selection of the MALDI matrix may help to solve this problem.

Our group developed a method, named "mass spectrometry assisted enzyme screening (MES)", by which enzyme activities in complex protein fractions can be measured with a mass spectrometer [5, 6]. In this study experiments are shown, which demonstrate the advantage of the MES method.

MATERIALS AND METHODS

All chemicals and enzymes were purchased from Sigma (Deisenhofen) if not stated otherwise. CNBr-activated-Sepharose 6MB beads were bought from Amersham Biotech (Freiburg). Porcine kidneys were obtained from the local slaughterhouse.

A. Covalent immobilization of proteins

Human renin, porcine renin, proteins extracted from porcine renal tissue and a protein lysate of hepatocytes were immobilized to BrCN-activated Sepharose Beads (Amersham Bioscience, Freiburg) according to the instruction manual. Briefly, for each experiment, 50 µL from the fractions (protein concentration 10 µg/µl) and 50 µl water as a control were each mixed 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. 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 double-distilled water and stored at 4°C.

B. Incubation of proteins with the reaction specific probes

For the detection of renin activity human renin substrate or porcine renin substrate (Bachem, Weil) was dissolved as reaction specific probes in HPLC-grade water (final concentration 10^{-5} mol/l). ACE activity was measured with angiotensin-I (Bachem, Weil) as reaction specific probe (final concentration 10^{-5} mol/l). To the immobilized proteins (10 µl beads) 20 µl of the probe-containing aqueous liquid was added. After defined incubation times 1 µl-aliquots (in triplicate) were removed and passed to the MALDI-MS analysis (C.).

C. MALDI-MS analysis of the reaction mixtures

For pipetting the reaction solutions to the MALDI-target a pipetting robot (Multiprobe II, Perkin Elmer, Rodgau) 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 and an AnchorChip^{TM} technology target were obtained from Bruker-Daltronics (Bremen).

One microlitre of the reaction mixture was applied on a 384-format AnchorChip^{TM} target in triplicate. Next, 1 µl of matrix solution (1:10 dilution of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in a 1:1 mixture of acetonitrile and water containing 0.1% TFA. The mixture was dried on the target before introduction into the mass spectrometer. Positively charged ions were analysed in the reflector mode of the MALDI mass spectrometer, using delayed ion extraction. Spectra were recorded with a 2-GHz data-sampling rate. Instrument high voltages were left on between analyses to ensure 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 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.

D. Measurement of enzymatic activity with a fluorospectro-photometer

As a fluorescent substrate the following renin substrate was used: Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg (Molecular probes). The substrate was dissolved in DMSO (final concentration 500 µM). For the determination of renin activity 4 µl of the substrate containing DMSO was mixed with 80 µl assay buffer (100 mM NaCl, 50 M Tris, pH 8, 1 mM EDTA) and 10 µl of the protein fraction.

Fluorescence was measured with a spectrofluoro-photometer (Fluoroscan, Thermo, Dreieich) at 355 nm (excitation) and 460 nm (emission).

E. Preparation of the protein extract from porcine renal tissue

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 30 min at 4°C. The pellet was discarded. One aliquot of the supernatant was used for the immobilization of the proteins.

F. Preparation and incubation of cultured human hepatocytes

Human liver tissue was obtained from partial hepatectomy due to liver metastases of a colorectal carcinoma. The isolated hepatocytes were seeded at a final density of 1.5 x 10^6 cells/ well into multi-well plates in Williams-E medium with insulin, dexamethason and 10% FCS. Prior to cell seeding, each culture plate was coated with collagen type I (Biochrom-Seromed, Berlin). When hepatocytes had attached firmly to the collagen matrix, culture medium was removed from the culture plates and hepatocytes were overlaid and cultured with Williams-E medium with insulin, dexamethason, but not with 10% FCS. Then, this medium was exchanged against HHMM (Human Hepatocyte Maintenance Medium) with 40 ng/ml HGF, 20 ng/ml dexamethason and 2,75 µg/ml insulin. The hepatocytes were cultured in the latter medium for 10 days [7]. The medium was exchanged every 48 h. After 10 days the hepatocytes were incubated for 5, 10, 20, 30 and 60 min with 10 nM glucagon dissolved in a glucose-free DMEM medium. For the control experiment, the hepatocytes were incubated with the glucose-free DMEM medium in the absence of glucagon. The incubation was stopped by washing the hepatocytes 3 times with an 0.9 % NaCl solution, cooled with ice. After washing, the cells were immediately frozen by adding liquid nitrogen. The cells were stored at -80°C. For the MES assay the hepatocytes were lysed by thawing the cells with the coupling buffer necessary for the immobilization of the proteins.

G. Measurement of kinase activity

For the detection of the cAMP-dependent kinase activity kemptide (Bachem, Weil) and ATP were dissolved in HPLC-grade water at final concentrations of 10^{-5} mol/l, 20 µl of the substrate mixture was added to 20 µl of the immobilized proteins. After defined incubation times 1 µl-aliquots (in triplicate) were removed and transferred to the MALDI-MS analysis. The signal in the resulting mass spectra indicated that kinase activity is 80 Da larger than the signal of non-phosphorylated kemptide.

RESULTS

The analytical procedure of the MES method is based on covalent immobilization of proteins to beads. By immobilizing proteins, 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. Locating a signal in the mass spectrum, which fits the molecular mass of the expected reaction product, validates the type of the enzymatic reaction.

Figure 1 gives an example of the detection of enzymatic activity with the MES method. In Fig. 1 the mass spectra of the reaction mixture of porcine renin substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) incubated for 1 h and 2 h with immobilized porcine renin and with immobilized human renin are shown. Porcine renin yielded the reaction product angiotensin-I. The intensity of the signal significantly increased with increasing incubation times. After two hours the signal intensity of porcine renin substrate had significantly decreased. In contrast human renin did not hydrolyze the porcine renin substrate. The mass spectra show no signals from angiotensin-I. The incubation of human renin substrate with immobilized human renin yielded signals from angiotensin-I in the mass spectra (data not shown). This experiment demonstrates that the high species-dependent reaction specificity of renin is maintained when using the MES method. The immobilization of the enzymes did not affect their enzymatic properties.

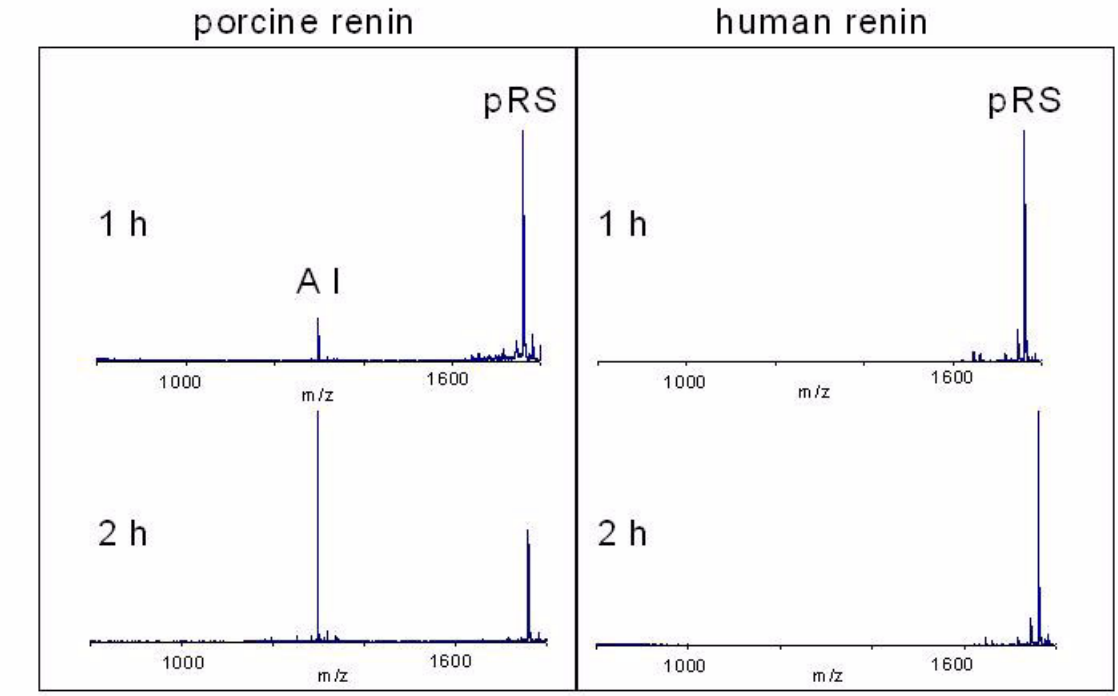

Figure 1. Detection of renin activity with the MES system. MALDI mass spectra of the reaction mixtures after 1 h and 2 h incubation of immobilized porcine renin and human renin with porcine renin substrate (pRS).
AI: Angiotensin-I.

The second experiment demonstrates the risk of obtaining incorrect positive results with substrates, which are labelled with fluorescent moieties. In this case a fluorescent analogue of renin substrate was used, representing the sequence of human renin substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn). This substrate is converted to angiotensin-I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by human renin but not by porcine renin (Table 1). However, incubating the fluorescent human renin substrate with a protein fraction from porcine renal tissue extract yielded a time dependent increase in fluorescence, representing the hydrolysis of the fluorescent human renin substrate. Therefore it must be assumed that the fluorescent substrate in the presence of the renal protein extract was hydrolyzed by another enzyme, which is not identical to renin. Because any cleavage of the peptide bonds of the fluorescent substrate yields fluorescence the resulting reaction products of the incubation of the fluorescent renin substrate with the renal protein extract must not be identical with the reaction products of human renin.

Table 1. Detection of renin activity in different protein fractions with a fluorophor-labelled renin-substrate Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg.
Fluorescence was measured with a spectrofluoro-photometer.

Incubation of human renin substrate with	increase in fluorescence
human renin	+
porcine renin	-
porcine renal protein extract	+

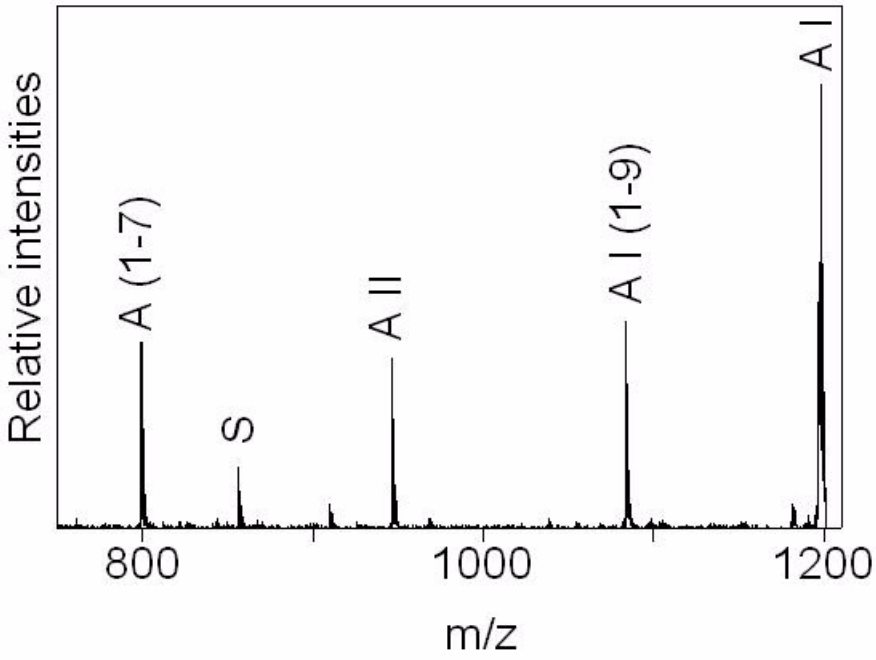

Figure 2. Detection of multiple proteolytic activities in a protein extract from porcine renal tissue. MALDI mass spectrum of the reaction mixture of the incubation of porcine renin substrate with the immobilized renal tissue proteins. A (1-7): Angiotensin (1-7); AII: Angiotensin-II; AI (1-9): Angiotensin-I (1-9); s: Internal standard.

In contrast with the MES method it is possible to detect the presence of several proteolytic enzymes in complex mixtures such as the renal tissue protein extract, as shown in Fig. 2. Here, the mass spectrum of the reaction products of the incubation of porcine renin substrate with immobilized proteins from a porcine renal tissue extract is given.

Beside angiotensin-I, the reaction product of renin, the signal of angiotensin-II represents the presence of an angiotensin-converting enzyme (ACE) activity, the signal of angiotensin-I (1-9) presumably demonstrates the presence of an ACE-2 activity and angiotensin (1-7) points to the presence of a neprilysin activity. This result clearly shows the advantage of the MES method. The mass spectrometric detection allows verification of the identity of the expected reaction products of an enzymatic conversion of the reaction specific probe. Furthermore unexpected enzyme activities can be observed via the identification of their reaction products.

A further advantage of MES is given by its high sensitivity. For the detection of ACE-activity a detection-limit for the MES system was determined, which was a factor of 1000 more sensitive than a comparable fluorescence-based ACE assay [6]. Because of its high sensitivity, the MES method was used for monitoring a cAMP-dependent protein kinase activity during the incubation of cultured human hepatocytes with glucagon (Fig. 3). For the MES kinase assay the peptide kemptide together with ATP was incubated with the immobilized proteins from the lysate of the hepatocytes. By the cAMP-dependent kinase activity kemptide becomes phosphorylated, which results in a second signal in the MALDI mass spectrum, which is 80 Da larger than the signal of kemptide.

Five minutes after starting the incubation with glucagon a significant increase in kinase activity was measured, reaching a maximum after 10 minutes. This experiment demonstrates that the MES method is suitable for the comparison of activities of low abundant enzymes, even in complex protein mixtures such as cell lysates.

Furthermore, the MES method closes the gap between transriptome-, metabolome-, and proteome-analysis, because MES yields additional information about the modulation of activities of enzymes, which are already expressed in the cell. Therefore MES is an additional tool for systems biology.

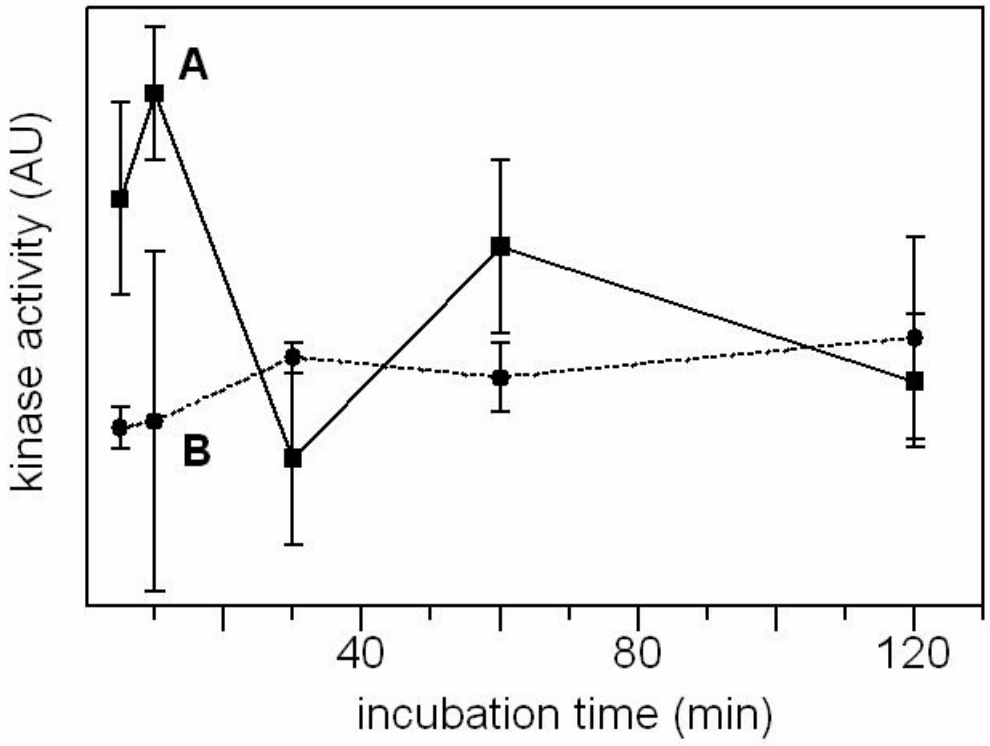

Figure 3. Time course of a cAMP-dependent kinase activity of hepatocytes stimulated with glucagon (A, solid line) and of unstimulated hepatocytes (B, dotted line). The relative enzyme activities were measured by the MES method using kemptide and ATP as substrates.

CONCLUSIONS

In conclusion the combined use of enzymology with MS provides much more detailed insight into the qualitative aspects of enzyme-catalysed reactions. The method is suitable for the analysis of the enzymatic conversion of low molecular weight substances as well as large biopolymers. Unlike the use of chromophore-labelled or radioactive substrates, there is little ambiguity as to the identity of the signal being measured. MS/MS analysis can offer additional structural information of the analyte being monitored. MS-based determination of enzymatic activities offers excellent accuracy, reproducibility and is especially well suited for assaying reactions that cannot be followed photometrically. The small sample size, minimal handling requirements, along with the potential for high-throughput, are further benefits of the combined use of enzymology with MS.

The MES approach enables the highly sensitive and reliable detection of enzymatic activities even in complex protein mixtures and therefore is a suitable tool for the determination of enzymatic activities in body fluids, cells or tissues.

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