MASS SPECTROMETRIC DETECTION OF ENZYMATIC BIOASSAYS

André Liesener

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Für meine Eltern

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Abbreviations

5-FSA	5-fluorosalicylic acid
5-FSAP	5-fluorosalicylic acid phosphate
AA	amino acid
Abu	aminobutyric acid
AC	affinity chromatography
ACESI-MS	affinity chromatography ESI-MS
Ach	acetylcholine
AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's disease
Ala	alanine
aP	alkaline phosphatase
Arg	arginine
ATCh	acetylthiocholine
BSA	bovine serum albumine
c	concentration
CDL	curved desolvation line
DIOS	desorption/ionization from porous silicon surface
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EALL	enzyme-amplified lanthanide luminescence
ESI	electrospray ionization
FIA	flow-injection analysis

Gly	glycine
HL_1	bis(2-pyridylmethyl)(2-acetoxyphenyl)amine
HPLC	high performance liquid chromatography
HTLC	high turbulence liquid chromatography
IC_{50}	inhibitor concentration, which induces 50% assay inhibition
K_I	inhibition constant
K_M	Michaelis-Menten constant
L	liter
LC	liquid chromatography
Leu	leucine
Lys	lysine
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MES	mass-spectrometry-assisted enzyme screening
MNBDA	4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole
MNBDH	4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole
MP-11	microperoxidase 11
MRM	multiple reaction monitoring
ms	millisecond
MS	mass spectrometry
NH_4Ac	ammonium acetate
nm	nanometer
PAH	phenylalanine hydroxylase
PG	protecting group
Phe	phenylalanine

PNA	*para*-nitroaniline
Pro	proline
psi	pound per square inch
RRP	relative reaction progress
RSD	relative standard deviation
RT	room temperature
s	second
SAM	self-assembled monolayer
SAMDI	self-assembled monolayers for MALDI
SIM	selected ion monitoring
S_P	product signal intensity
S_S	substrate signal intensity
TIC	total ion current
u	units
UV	ultraviolet
v/v	volume-to-volume ratio
V	volt
Vpp	volt per peak
Val	valine
vis	visible
v_{max}	maximum velocity

Chapter 1

Introduction

1.1 Introduction and scope

Enzymatic conversions play a crucial role not only in the regulation of all processes of life. As biocatalysts in industrial processes or as targets in drug discovery, enzymes originating from natural and synthetic sources are getting more and more important. Generally, the interest in not only identifying an enzyme but also characterizing its catalytic functionalities is high. Other important objects of research are potent enzyme inhibitors. These compounds are of special interest for the development of new pharmaceuticals acting highly selective on their target enzymes. Therefore, it is of utmost importance to develop rapid and sensitive methods for the elucidation of enzymatic catalysis mechanisms and the characterization of enzymatic activities.

Generally, enzyme-catalyzed reactions are following the mechanism depicted in figure 1.1.

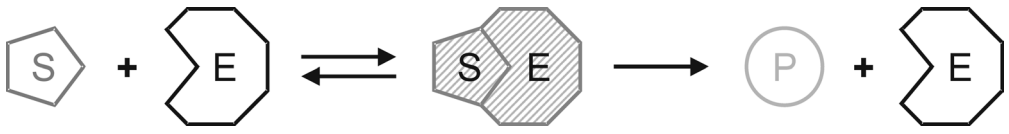

Figure 1.1: Reaction scheme of an enzyme-catalyzed conversion of the substrate S to the respective product P.

In the first step, a suitable substrate enters the active site of the enzyme where it is bound, thus forming an enzyme-substrate complex. Since the formation of the complex is an equilibrium reaction, the substrate is either converted by the enzyme to the respective product form or released from the active site, thus breaking the complex. If the substrate is converted, the product is released from the complex in the last step, thus allowing another substrate molecule to enter the enzyme's active site [1].

Most enzyme assays are based on the change of spectroscopic properties during the conversion of the substrate used. Detection can be done by UV/vis absorption or fluorescence spectroscopy. Due to the fact that most natural occurring substrates do not possess any distinct fluorescent or chromophoric moieties, it is necessary to introduce those functionalities synthetically into the molecular structure. These modifications might alter the enzyme recognition of the substrate compound, thus changing the kinetic behavior significantly [2]. To circumvent this, the detection can be based on a secondary reaction coupled to the enzymatic conversion in question, in which an optically detectable signal is generated. Another widely used detection technique is based on the release of radioactive labels during the enzyme-catalyzed reaction. In this case, the change in radiation intensity is the signal to be evaluated. This method is advantageous, since the radioisotopically changed substrates are chemically identical to the natural occurring compounds, thus ensuring similar enzymatic kinetics. Unfortunately, the availability of radioactive-labeled substrate compounds is often limited. In that case, laborious synthetic work with radioactive material is required to obtain the

desired compounds. An additional problem is the generation and disposal of radioactive waste.

Therefore, the demand for a label-free, non-radioactive assay scheme is high. As proposed by Northrop and Simpson [3], with its continuous technological advancements, mass spectrometry (MS) can be a viable alternative to established detection methods. Since MS is independent from the spectroscopic properties of the analyte molecules, no modification is required and the native substrate compounds can be utilized for enzyme assays. Another advantage of mass spectrometric detection is the general possibility to monitor simultaneously the fate of multiple analytes during an enzyme-catalyzed reaction.

Since the first approaches to employ MS as means of detection bioassays by Henion and co-workers [4], only a surprisingly small number of papers have been published about this topic, but the interest in this field is growing steadily. In **chapter 2**, the development and trends in the employment of MS detection for the monitoring of enzymatic conversions is described. The focus is laid on assay schemes using the so-called “soft” ionization techniques electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), and advantages and disadvantages of both methods are discussed within this chapter.

The goal of this thesis was to develop assay schemes for the rapid screening of relevant activities in complex biological samples such as snake venom. As sample of interest, the venom of *Bothrops moojeni* was chosen. The use of ESI-MS as means of detection seemed to be most promising due to its compatibility with liquid-phase reactions and its inherent feasibility for multiplexed analyses.

In **chapter 3**, different strategies for the employment of ESI-MS as means of detection in the monitoring of enzymatic reactions are presented. With respect to independent reference method, a fluorescence-based detection scheme was developed.

The possibility of monitoring two independent enzyme-mediated conversions simultaneously in one single experiment for the quantitative determination of the respective enzymes is discussed in **chapter 4**. As means of detection, ESI-MS and fluorescence were selected, and both detection methods were directly compared.

The development of a multiplexed assay scheme for the simultaneous monitoring of multiple substrate conversions is described in **chapter 5**. The assay scheme allowed the simultaneous enzymatic activity assessment for a protease towards seven different substrates. The results of the multiplexed MS-based assay were validated by single-substrate UV/vis-absorption-based assays.

Based on this multiplexed approach, an assay scheme for the rapid identification and characterization of the proteolytic activities in the venom of *Bothrops moojeni* was developed (**chapter 6**). The generation of enzymatic activity maps as fingerprints for the venom fractions allowed the identification of model-like activities by comparison with the activity patterns of several model proteases.

In **chapter 7**, the possibility to employ naturally occurring substrates in MS-based assays was used to develop an acetylcholinesterase assay. This assay was employed in the screening for acetylcholinesterase-inhibiting activities in the fractions of the snake venom. The inhibition potential was quantified in relation to a known acetylcholinesterase inhibitor.

The potentially deteriorating influence of matrix components on the signal response in ESI-MS-based detection is discussed in **chapter 8**. High turbulence liquid chromatography was introduced as means to efficiently minimize these matrix effects.

General conclusions and future perspectives of using ESI-MS-based assay schemes for the determination and identification of catalytic or inhibiting activities in complex natural samples are discussed in **chapter 9**, which concludes this work.

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Chapter 2

Monitoring Enzymatic Conversions by Mass Spectrometry[‡]

This chapter highlights recent advances in the application of electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry to study enzymatic reactions. Several assay schemes for different fields of application are presented. The employment of MS as means of detection in pre-steady-state kinetic studies by rapid-mixing direct analysis and rapid-mixing quench flow techniques is discussed. Several steady-state kinetic studies of a broad range of different enzymatic systems are presented as well as enzyme inhibition studies for various target enzymes. A particular focus was directed to multiplexed assays, which monitor the conversion of several substrates simultaneously in one experiment. This assay type was used for competition studies and enzymatic activity screening as well as for diagnostic purposes in clinical chemistry.

Generally, it can be concluded that mass spectrometry offers an intriguing alternative as detection methodology in enzymatic bioassays. Its applicability for monitoring the conversion of naturally occurring substrates and its overall versatility make MS a promising tool for the study of enzyme-catalyzed processes.

[‡] A. Liesener, U. Karst, submitted for publication in *Anal. Bioanal. Chem.* **2005**.

2.1 Introduction

As proposed by Northrop and Simpson [2], mass spectrometry (MS) with its continuous technological advancements can be a viable alternative to established detection methods for enzymatic bioassays. Since MS is independent from the spectroscopic properties of the analytes, no modification is required and the native substrates may be applied for enzyme assays. Another advantage of mass spectrometric detection is the general possibility to simultaneously monitor the fate of multiple analytes during an enzyme-catalyzed reaction.

In this chapter, the development of MS-based detection schemes in enzymatic bioassays shall be highlighted. The focus is directed to assays employing the so-called “soft” ionization techniques electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI).

2.2 ESI-MS-based assay schemes

2.2.1 Development of ESI-MS assays

Since the introduction of ESI-MS as a tool for the mass spectrometric analysis of large biomolecules by Fenn and co-workers in 1989 [3], this ionization technique has become the predominantly used method for coupling of liquid chromatography (LC) to MS. ESI-MS plays an important role in a wide range of different applications such as analyte quantification, structure elucidation of biomolecules as well as protein-protein- and protein-ligand interaction studies.

The first approaches of employing ESI-MS for kinetic studies of enzymatic conversions were undertaken in 1989 by Henion and co-workers [4]. They coupled a closed reaction vessel to the ESI-interface of a triple-quadrupole mass spectrometer, monitoring the ongoing reactions on-line in a continuous-flow experiment (see figure 2.1).

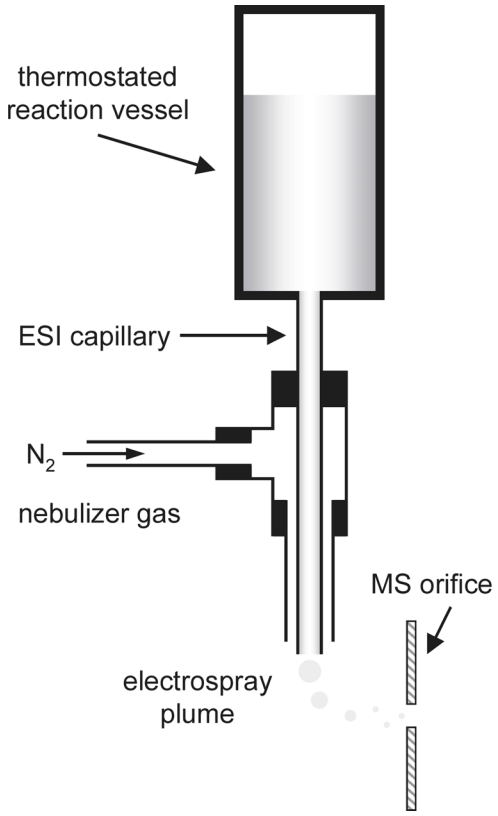

Figure 2.1: Schematic set-up of the continuous-flow ESI-MS employing a thermostated reaction vessel, which is directly coupled to the ESI-interface [4].

Thus, they determined the Michaelis-Menten constant (K_M) of the lactase-catalyzed hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside and followed the enzymatic degradation of the peptide dynorphin 1-8 by α-chymotrypsin and leucine aminopeptidase in real time. In another paper by the same group, an off-line approach was introduced employing a LC/ESI-MS system for the determination of the kinetic parameters of the hydrolysis of dinucleotides by pancreatic ribonuclease A and the β-galactosidase-catalyzed hydrolysis of lactose following the substrate consumption [5].

In 1997, Siuzdak et al. introduced ESI-MS as means of screening for inhibitory activities [6]. A small library of potential inhibitors were assayed individually against β-1,4-galactosyltransferase and the appropriate substrates. The reaction was carried out off-line and quenched with methanol after a defined time. The crude reaction mixture was directly injected into an ESI-MS system and the amount of product formed was quantified using an internal standard. The inhibitory activity of the library compounds was assessed by comparison to the amount of product formed in a reference assay without the presence of an inhibitor. By variation of the inhibitors concentration, the inhibitor concentrations, which induce a 50% assay inhibition (IC_{50} values) for the three most potent inhibitors could be determined.

Since these first studies on the feasibility of MS detection in enzyme assays for either the determination of kinetic parameters or inhibitory activity, the interest in this field has been growing, thus resulting in a strongly increasing number of publications over the last years. Several different enzymatic systems have been investigated this far, and different technical approaches to

employ MS as means of detection in enzymatic bioassays have been made. Bothner et al. investigated the kinetic parameters of glucosidase- and lipase-catalyzed hydrolysis reactions and found significant differences in the kinetics when using chromophor-labeled and natural substrates [7]. These results underline the advantage of the use of MS, when studying physiologically relevant enzymatic reactions.

The group of Toyokuni and co-workers investigated the catalytic activity of α-1,3-fucosyltransferase using direct flow-injection analysis (FIA) coupled with an ESI triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode [8]. Comparative experiments with radioactivity-based assays showed good agreement of the obtained kinetic parameters. Using the possibility to monitor the concentration of an inhibitor present in the reaction mixture simultaneously with the product formation, a competitive inhibition mechanism was proposed [9]. It was furthermore possible to differentiate the kinetics of two structurally closely related potential inhibitors [10]. Thus, Toyokuni et al. could demonstrate the feasibility of ESI-MS as tool for unraveling complex inhibition mechanisms.

In 2001, Ge et al. presented a general strategy for the determination of enzyme kinetic constants using ESI-MS [11]. As model enzyme, glutathione S-transferase was used. The reactions were carried out off-line in a vessel, and aliquots were taken from the reacting mixtures and quenched in an excess of methanol. The crude quenching mixture was directly analyzed using FIA/ESI-MS. Quantification of the product was achieved in the selected ion

monitoring (SIM) mode by using an internal standard, which was added to the quenching solution. This allows the use of compounds, which are structurally very similar to the substrate. The enzymatic reaction is not disturbed, thus increasing the precision of quantification. The actual concentration of the product in the sample and the initial reaction velocity were calculated by using a so-called single-point normalization factor. This normalization factor represents the ratio of signal intensity of the product and the concentration of the internal standard to the signal intensity of the internal standard and the product concentration. This factor can be determined by two measurements and renders the use of a complete calibration curve for the quantification of the product obsolete, thus significantly reducing the number of samples needed to obtain kinetic data about an enzymatic conversion by means of ESI-MS. The validity of the approach was demonstrated by comparing the results obtained by the ESI-MS assays with those from UV-absorbance assays.

In further work from the same group, Pi et al. demonstrated the use of this strategy for investigations on the kinetics of yeast hexokinase and a bacterial carbohydrate sulfotransferase (NodST) [12, 13]. They determined the relevant kinetic parameters K_M and v_{max} as well as the inhibition constant K_i for the product formed in the respective reactions. These findings allowed the conclusion of a hybrid double-displacement, two-site ping-pong mechanism of the NodST-catalyzed sulfuryl transfer [14]. With this, Pi et al. could demonstrate the use of the MS-based detection approach in elucidating complete reaction pathways and catalytic mechanisms of an enzymatic system.

Following the strategy proposed by Pi and co-workers, Deng et al. developed an HPLC-MS-based assay scheme for an inhibitor screening procedure [15]. The target enzyme was UDP-*N*-acetylmuramyl-L-alanine ligase (MurC) and enzymatic activity was determined by LC-separation of the reaction mixture followed by quantification of the product formed by SIM-mode MS. They compared the efficiency of this MS-based assay with a standard optical, malachite green assay finding significant lower background values (0.2 % to 26 %), a ten-fold higher sensitivity, a lower limit of quantification by a factor of five (0.2 μM to 1.0 μM) and a four-fold wider linear range.

Zea and colleagues employed the MS-based detection scheme in studies of the kinetics of sugar phosphorylases using rabbit muscle phosphorylase *b* as model enzyme [16] and of sugar nucleotidyltransferases using enzymes from several sources as model systems [17, 18]. In both cases, the possibility of directly monitoring substrate and product was used to circumvent the problem of a secondary coupled assay, which is commonly used for investigations on these types of enzymes. This is especially disadvantageous when screening a variety of different possible substrates, which might not be detected correctly by the secondary assay, or in inhibition studies, where the inhibitor might interfere with the detection assay. With MS detection, it was possible to evaluate the enzymatic activity towards a number of different potential substrates and to obtain information about the inhibition activity of a number of putative inhibitors.

The groups of Hines et al. and Fabris developed assays for assessing the activity of ricin A chain, a *N*-glycosidase of interest as a potential therapeutic drug and possible bioterrorist agent [19]. The ricin A chain acts on ribosomal RNA strands releasing an essential adenine, thus inhibiting protein synthesis in cells and finally leading to cell death. Hines and co-workers employed a LC-MS assay detecting the adenine released during the enzymatic reaction. The LC step before MS detection was necessary in order to achieve a satisfactory sensitivity. By separation of the analyte from the reaction mixture constituents, the ionization suppression of adenine by ricin or the substrate RNA could be significantly reduced [19]. Fabris used a FIA-MS approach detecting the depurinated RNA strand that is the product of the reaction. He demonstrates the possibility of generating quantitative data by using the molar fractions of product and substrate calculated from their relative ion intensities. This approach renders the use of internal standards and calibration curves unnecessary, but it is only possible when investigating products and substrates with similar masses and structures, thus ensuring comparable ionization behavior [20].

An automated direct-injection FIA approach without the need for off-line sample quenching was developed by Steinkamp et al. in order to monitor an esterase-catalyzed ester hydrolysis [21]. The method was used for the validation of a time-resolved fluorescence assay scheme and was found to be of comparable sensitivity. De Angelis and co-workers employed a FIA-MS assay for studies on the activity of α-chymotrypsin in water in the presence of cetyltributylammonium bromide. Assays carried out with and without addition

of the surfactant showed a significant increase of enzymatic activity in the presence of the surfactant. MS detection allowed in this case the first studies on the superactivation of α-chymotrypsin towards a natural substrate (substance P) [22].

A true on-line assay scheme for the determination of inhibition activities in complex natural samples such as red clover and fungi extracts was realized by de Boer and co-workers [23]. A LC separation step is coupled to a continuous-flow enzymatic assay, which is detected by means of ESI-MS.

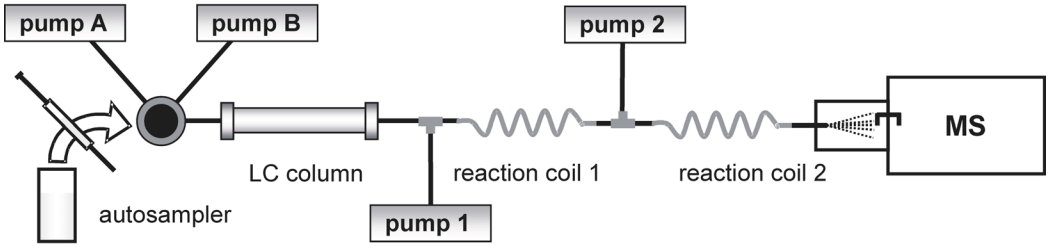

Figure 2.2: Schematic set-up of the continuous-flow on-line inhibition assay with ESI-MS detection; pumps A and B are providing the eluent for the HPLC separation of potential inhibitors. Pump 1 delivers a constant stream of enzyme solution; pump 2 delivers a constant stream of substrate solution [23].

A constant stream of enzyme and substrate solutions is mixed into the LC eluent resulting in the formation of a constant amount of product (reaction coil 2), which is detected continuously in the mass spectrometer. Reaction coil 1 is used to allow an inhibitor eluting from the column to interact with the enzyme prior to entering the assay zone. When an inhibitor elutes from the LC column into the assay zone, the product formation is slowed down, and a drop in

product signal intensity is observed. This change in signal intensity can be quantified and is a measure of the respective inhibitor´s efficiency. As model enzyme, cathepsin B was selected. The assay was tested on a spiked red clover extract and a fungi extract in order to identify inhibitors. Validation of this assay scheme was performed using four known inhibitors and determining their IC_{50} values in the flow-injection mode. This combination of chromatographic separation and biological signal evaluation carries a great potential for the use in inhibition screening of natural compounds.

2.2.2 Multiplexed ESI-MS assay schemes

All of the previously described assay schemes were single-enzyme-single-substrate assays. The first approaches to a multiplexed analysis were presented by Gerber and colleagues, studying two independent single-enzyme-single-substrate reactions in parallel. They developed an assay scheme, which allowed the parallel assessment of multiple enzymatic activities in cell lysates, thus providing a tool for the diagnosis of enzyme deficiency related diseases, e.g., the Sanfilippo syndrome. The enzyme assays were carried out off-line employing synthetic target-enzyme-specific substrate conjugates, where the substrate moiety is covalently attached to an affinity tag via a linker. The affinity tag allows the highly selective extraction by means of affinity chromatography. During the enzymatic conversion, the substrate conjugate is modified resulting in a change of molecular mass. After quenching of the reaction, the labeled substrate and product species were extracted by means of affinity chromatography and the eluent was injected into the ESI-equipped mass spectrometer. Quantification was carried out by

use of an internal standard. Enzymatic reaction rates were calculated from a single point determination [24-26]. This ACESI-MS (affinity chromatography electrospray ionization mass spectrometry) named assay scheme was employed in further work from the same group leading to the development of a novel efficient newborn screening for deficiencies in five lysosomal enzymes causing Fabry, Gaucher, Krabbe, Niemann-Pick A/B and Pompe disease [27, 28]. Li et al. presented an ACESI-MS based approach for determining phosphomannomutase and phosphomannose isomerase activity. Since the products formed by these enzymes are isomeric to the educts, a direct mass spectrometric analysis is not possible. Therefore, a secondary enzymatic reaction had to be used, converting the products from the first assay into compounds of different molecular masses. With this approach, the monitoring of isomerase-catalyzed reactions by means of ESI-MS could be demonstrated [29]. A method for automating the ACESI-MS assays was presented by Ogata et al., thus allowing this assay scheme to be used for high-throughput clinical applications [30].

Basile et al. developed a direct flow-injection ESI-MS assay, which allowed the *in vivo* differentiation of bacteria by their aminopeptidase activity profile. Living bacteria were immobilized on a filter and incubated with a mixture of four target-enzyme-specific substrates. Upon enzymatic activity towards a certain substrate, a unique tag is released from the respective compound. Following the incubation, the reaction mixture is filtered, thus retaining the bacteria and the clear filtrate containing a mixture of non-reacted substrates and products is directly injected into the mass spectrometer. The conversion

rate of each substrate is determined. Each bacterium was found to exhibit different enzymatic activities towards the substrates resulting in unique activity profiles [31]. In a similar approach, Yu et al. developed a multiplexed assay scheme for the functional characterization of glycosidases. In this approach, a library of potential substrates, which differ in their molecular masses, is incubated with the enzyme of interest and the reaction mixture is directly analyzed by ESI-MS. Activity profiles can be generated, thus characterizing the enzymes studied. The authors used a library containing nine different carbohydrate substrates [32]. These multiplexed assay schemes open a wide range of screening options.

A study on the characteristics of a two-enzyme and three-enzyme tandem reaction involved in the multi-step biosynthesis of novobiocin was published by Pi et al. in 2004. Since multiple species can be simultaneously detected in ESI-MS, it was possible to generate detailed time-resolved reaction profiles quantifying the concentrations of the starting substrate, intermediately formed products and the final product. These profiles were used for the kinetic characterization of each step in the multienzyme-catalyzed formation cascade [33].

An assay scheme studying the competitive conversion of two substrates by one enzyme was presented by Zea and co-workers. The enzyme in question was archaea glycogen synthase. Glycogen synthases catalyze the carbohydrate polymerization to form glycogen/starch. Enzymes from animal/fungal and plant/bacterial sources are using different substrates for the

biosynthesis of the same product. The archaea glycogen synthase was found to convert both substrate species. The enzyme-catalyzed polymerization was monitored by following the consumption of the substrate. In order to determine the kinetic parameters for the conversion of both substrates, the concentration of one compound was varied in the assays, while the concentration of the other was kept constant. This competitive assay revealed that both substrates are serving as competitive inhibitors of the other. These findings allowed the conclusion that both substrates are converted in the same binding pocket of the enzyme [34]. This example clearly indicates the potential of MS-based enzymatic assays to be used for the elucidation of the functionalities and mechanisms of enzymes.

Pi et al. presented a multiplexed assay scheme for the simultaneous determination of multiple substrate specificities of one enzyme in a single experiment. The bacterial sulfotransferase NodH was used as model enzyme with four chitooligosaccharides as substrate compounds. Kinetic parameters were determined for both the single-substrate experiments and the multiplexed assays, respectively. The calculated values for both types of assays were found to be in good compliance, thus cross-validating the approach [35]. This method for the parallel determination of kinetic parameters helps to significantly decrease the number of samples to be run, thus additionally reducing the amount of enzyme needed for the study.

2.2.3 Pre-steady-state kinetics by ESI-MS

The determination of kinetic parameters and the conclusions about reaction pathways and catalytic mechanisms in the aforementioned studies were all derived from steady-state experiments. A more direct approach to investigate the actual reaction mechanism is provided by pre-steady-state kinetic experiments, in which information about transient intermediates, their chemical structure or rate constants of individual reaction steps can be obtained. The time window for pre-steady-state experiments is rather small (in the order of milliseconds to seconds), thus efficient mixing and a fast analysis is required [36].

Zechel et al. presented the first on-line time-resolved ESI-MS approach to accurately study the pre-steady-state kinetics of an enzymatic conversion. Therefore, they monitored the fate of a transient covalent enzyme-substrate intermediate complex. The model enzyme used in this study was xylanase with β-xylobioside as substrate. The experimental set-up comprised two syringe pumps for the constant delivery of the enzyme and substrate solution, a mixing tee and a reaction capillary coupled to the ESI-interface.

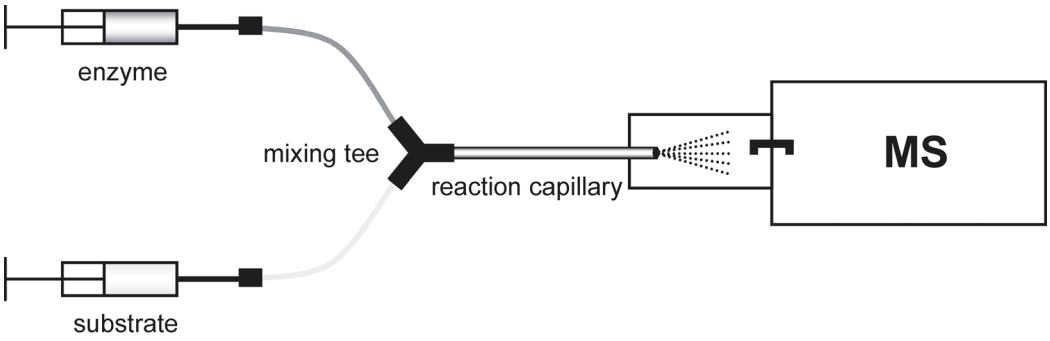

Figure 2.3: Schematic set-up of the rapid-mixing device for on-line pre-steady-state kinetic measurements. One syringe provides a constant stream of enzyme solution, the other of substrate solution. The reaction is initiated by rapid mixing in the mixing tee. Variation of reaction times is achieved by changing the length of the reaction capillary, which is directly connected to the ESI interface [37].

Different reaction times were realized by changing the length (volume) of the reaction capillary. The reaction was considered to be stopped upon nebulization of the reaction mixture in the interface. With this approach a time resolution in the range of tens of milliseconds could be realized. In order to validate this method, the resulting pre-steady-state kinetic parameters were compared to those calculated from stopped-flow UV-absorption experiments and found to be in excellent agreement [37]. Turbulent flow conditions within the system in order to prevent a broadening of the age distribution had not to be established. Exact kinetic measurements even with high time resolution are well possible in the laminar flow regime, as shown by Konermann [38].

With an experimental set-up similar to the rapid-mixing continuous-flow ESI-MS system described above, Li and co-workers were able to identify an unstable non-covalent hemiketal phosphate intermediate complex in the catalytic cycle of 3-deoxy-D-manno-2-octulosonate-8-phosphate synthase [39]. Attwood and Geeves studied the trypsin-catalyzed hydrolysis of a model substrate. The kinetic parameters were calculated from the monitoring of substrate consumption, product formation and changes in free enzyme and acyl-enzyme intermediate concentrations. The resulting values were found to be in good agreement with parameters calculated from UV-absorption based assays [40].

The variation of the reaction time by changing the flow rates of the reactant solutions might result in changes of the MS-signal response, thus possibly leading to erroneous results. For this reason and as changing the ion capillary is rather time consuming and inflexible, an alternative approach was made by Wilson and Konermann. They derived a capillary mixer with an adjustable reaction chamber for the time-resolved ESI-MS measurements [41].

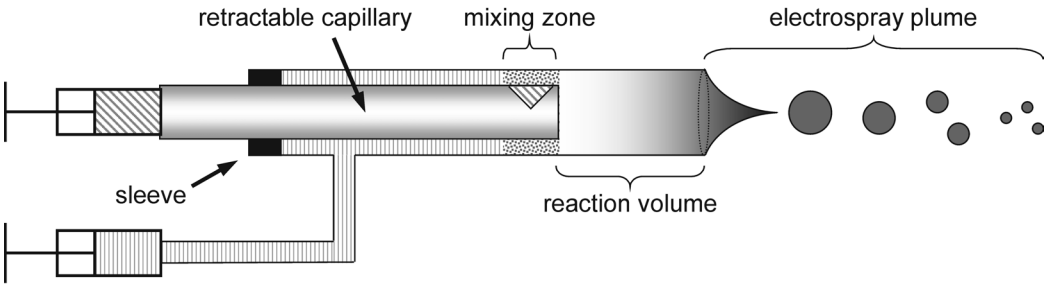

Figure 2.4: Schematic set-up of the rapid-mix device with variable reaction volume for on-line pre-steady-state kinetic measurements. One syringe provides a constant stream of enzyme solution, the other of substrate solution. The reaction is initiated upon rapid mixing at the outlet of the inner capillary. Variation in reaction times is achieved by moving the inner capillary, thus changing the reaction volume between the outlet and the ESI-interface [41].

Reactant solutions are delivered by two syringe pumps at a constant flow rate. Syringe 1 is connected to a retractable capillary, which is mounted in a larger capillary connected to syringe 2. By moving the inner capillary backwards, the reaction volume can be enlarged, thus prolonging the reaction time. The reaction is stopped upon nebulization of the reaction mixture in the ESI-interface. With this set-up, a time resolution in the range of a few milliseconds can be realized. Wilson and Konermann employed the described device with the addition of a make-up flow after the reaction zone in mechanistic studies of the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate and bradykinin. The resulting rate constants for the pre-steady-state kinetics of the conversion of *p*-nitrophenyl acetate were found to be in excellent agreement with data derived from UV-absorption-based stopped-flow measurements. For the conversion of bradykinin, steady-state kinetic measurements were carried

out to determine the turnover number and K_M-value [42]. This methodology promises to be an extremely valuable tool in the kinetic studies of enzyme-catalyzed reactions, allowing the rapid and accurate assessment of the reactions mechanisms by either steady-state or by pre-steady-state experiments.

2.3 MALDI-MS-based assay schemes

2.3.1 Development of MALDI-MS assays

Since the introduction of matrix-assisted laser desorption/ionization as a new tool in biomolecular mass spectrometry by Karas and Hillenkamp in 1988 [43, 44], this ionization technique was employed in the analysis of proteins, peptides, oligonucleotides and polymers in a wide range of applications. Several approaches using MALDI-MS for the monitoring of enzymatic conversions have been made [7, 45 – 53].

While MALDI-MS offers certain advantages regarding the high tolerance against salt or buffer content and contaminants in the sample, quantification is often problematic. Due to inhomogeneous distribution of analytes within the matrix crystal, shot-to-shot reproducibility (generation of so-called “hot spots”) and sample-to-sample reproducibility remain mostly poor. Several factors like matrix compound selection, pH value of sample solution, ratio of matrix to analyte molecules, target surface and sample drying method are critical for the crystallization process and have to be carefully optimized [54].

There have been some approaches to the accurate quantification of substrates and products generated in an enzyme-catalyzed reaction without the addition of an internal standard. The general concept is based on the assumption that the signal responses for the product and substrate are similar. Therefore, the sum of the signal intensities at each time point should be identical and equal the signal intensity for the starting concentration of the substrate. The second assumption is a strictly linear relation between signal intensity and concentration over the whole range. With those two assumptions, one can calculate the actual concentration of a compound from the substrate or product signal intensity divided by the summed intensities of product and substrate signal [54 – 56]. Since the aforementioned assumptions are only rarely met, this approach remains semi-quantitative and is not sufficient to accurately determine the concentrations of reactants in a reaction mixture. To achieve a sufficiently reliable quantification, the use of an internal standard, which is chemically as similar as possible to the analyte, is inevitable.

Based on this idea, Kang et al. developed a high-throughput protocol for the automated determination of enzymatic activities. The enzyme model system used was the lipase-catalyzed conversion of *rac*-1-phenylethylamine. In order to quantify reliably, the deuterium-labeled substrate was added to the matrix as internal standard. It was found that quantification works best for the ratio of analyte to internal standard between 0.2 and 5, leading to a relative standard deviation of <5%. To circumvent the problem of crystal inhomogenities, 100 acceptable spectra were measured from seven to ten different positions of

one sample spot and averaged. The method was validated with a gas chromatography-based quantification scheme and was found to be in good compliance [57].

Bungert et al. investigated the potential of the MALDI-MS-based assay scheme for the quantification of low molecular weight products and substrates directly from reaction mixtures. As model systems, the glucose oxidase-based conversion of glucose to gluconolactone and the carboxypeptidase A-mediated cleavage of hippuryl-L-phenylalanine were chosen. Time resolved reaction profiles for both conversions were obtained by simultaneous determination of the respective substrate and product concentrations without the need for time-consuming sample preparation steps. The results were compared with those from a standard UV absorbance-based assay and were found to be in good agreement [58]. In another study by Bungert and colleagues, the automated MALDI-MS assay detection scheme was further refined. In order to minimize the negative effects on the quantification by sample spot inhomogenities, a liquid ionic matrix was employed instead of using a crystalline solid matrix. The method was applied to screen the enzymatic activity of ten pyranose oxidase variants towards glucose. Reactions were carried out independently and aliquots were quenched at four different time points. Each sample was mixed with the ionic liquid matrix (2,5-dihydroxybenzoic acid / pyridine) containing ^{13}C-labelled glucose as internal standard and spotted on the target. MALDI-MS analysis was performed in fivefold generating reaction profiles by the simultaneous determination of product and substrate concentrations for each enzyme variant. The reaction

profiles could be used to sort the enzyme variants into five different classes [59]. This study shows clearly the suitability of the MALDI-MS-based assay schemes for the application in rapid, enzyme activity screening procedures.

2.3.2 DIOS-MS-based assay schemes

A general problem when quantifying low molecular weight compounds by means of MALDI-MS is the potential interference of matrix signals with the analyte signals. To circumvent this problem, Wei et al. developed a matrix-free strategy based on the pulsed laser desorption/ionization of molecules from a porous silicon surface (DIOS) [60]. The analytes in solution are spotted on the silicon target and evaporated to dryness. Analysis can then be performed by using standard MALDI-MS equipment. The fact that little to no fragmentation is induced by the desorption/ionization process is especially advantageous.

A first application of the DIOS-MS method to monitor enzyme-catalyzed reactions was presented by Thomas et al. in 2001 [61]. The enzymatic systems investigated comprised of a glucosidase, a lipase and an esterase, namely acetylcholinesterase and their respective substrates. Reactions were carried out directly on the target surface. The reactions were stopped by evaporation of the solvent and direct DIOS-MS analysis of the dried reaction mixture could be performed. Acetylcholinesterase was assayed with its naturally occurring substrate acetylcholine. Quantification was performed by using deuterated choline as internal standard. A time-resolved reaction profile was generated by plotting the choline formation over the course of time.

Inhibition of acetylcholinesterase by three different inhibitors was studied in three independent reactions on one target plate, allowing to screen the inhibiting activities within 15 minutes including sample preparation time. The inhibition potentials of each inhibitor were found to be in accordance with their known K_i-values. The assays of mannosidase II were carried out on differently modified target surfaces, showing best signal responses of the carbohydrates when using an oxidized silicon surface. Thus, tailoring of the surface properties might be advantageous for DIOS-MS analysis of different reaction mixtures. A further advantage, as demonstrated by this study, is the possibility of protein identification following the functional characterization of the enzyme.

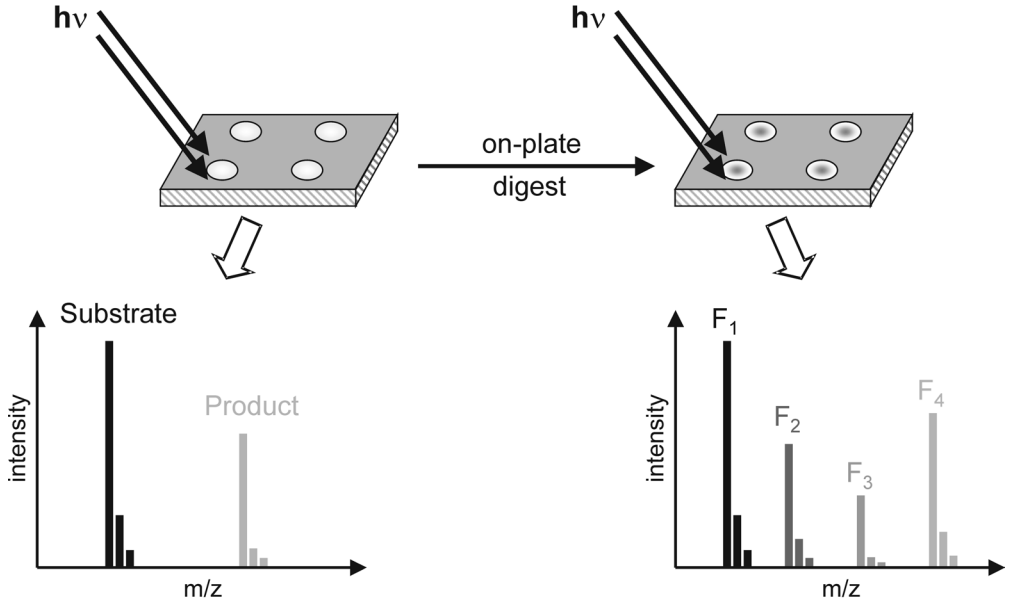

Figure 2.5: Scheme of a combined enzymatic activity and enzyme identification assay based on DIOS-MS. In the first step, information about the activity is generated by assessing the product formation. After on-plate digestion of the enzyme, the generated peptide fragments (F_1 to F_4) are determined.

In the first assay, the activity of an enzyme is determined by following the substrate consumption and product formation. Since no matrix components are present in the sample spot, the immobilized enzyme is then directly incubated with site-specific proteases, thus being digested. The digest is afterwards analyzed again by means of DIOS-MS, thus generating a protein-specific peptide mass map. The protein fragments generated can be used for the correct identification of the protein in combination with a database search [61, 62].

Wall et al. presented a study using the DIOS-MS-assay approach for the determination of the kinetic parameters of the acetylcholinesterase-catalyzed deacetylation of acetylcholine [63]. In a second set of experiments, the IC_{50}-value for the inhibitor tacrine was determined. The results from the DIOS-MS-based assays were compared to those generated by an LC/ESI-MS/MS assay scheme. The good correlation of the values confirms that DIOS-MS is a suitable tool for the rapid quantitative analysis of enzyme kinetics and inhibitions.

Functionalization of a DIOS-target by immobilization of trypsin onto the surface was demonstrated by Xu et al.. The enzyme was immobilized using 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) as coupling agent following an amino-functionalization of the silicon wafer. The immobilized enzyme retained its bioactivity and the kinetic parameters for the trypsin-catalyzed proteolysis of N_{α}-benzoyl-L-arginine ethylester were determined. The value for v_{max} was found to be lower than for free trypsin, which indicates a slight loss of activity

due to the immobilization of the enzyme. The trypsin-functionalized DIOS-target was used for peptide mapping analysis of two model proteins. Solutions of cytochrome c and bovine serum albumin (BSA) were incubated on the target and after evaporation to dryness directly analyzed by means of DIOS-MS. The signal intensity of the peptide fragments generated was found to be low. This might be due to the surface modification. Therefore, a small amount of matrix (α-cyano-4-hydroxycinnamic acid) was added to increase the signal response significantly. In the presence of matrix, 19 peptide fragments could be assigned with a sequence coverage of 98.6% for cytochrome c and 54 peptide fragments could be assigned with a sequence coverage of 65.6% for BSA [64]. This demonstrates the versatility of the DIOS-MS approach to be "converted" to a MALDI-MS scheme for enhancing signal intensities, when needed.

Shen et al. published an automated DIOS-MS-based approach as screening assay for enzymatic activities and enzyme inhibitors in 2004 [65]. The so-called "DIOS-MS plate reader assay" was employed in an enzyme activity screening, searching for new enzymes with a similar activity as phenylalanine hydroxylase (PAH). PAH is a biologically relevant enzyme converting L-phenylalanine to L-tyrosine. Determination of the respective kinetic parameters as measure of catalytic activities was achieved by varying the substrate concentration and monitoring the rate of product formation and substrate consumption. Quantification of product and substrate was carried out using deuterium-labeled phenylalanine and tyrosine as internal standard. In a second set of experiments, the DIOS-MS plate reader assay was

employed in a screening for potential inhibitors of AChE. The library of potential inhibitors comprised more than 900 compounds including some known inhibitors as references. Inhibition activity was assessed by calculating the ratio of signal intensities of substrate (acetylcholine) to product (choline), with high values indicating a high level of inhibition. All enzymatic reactions in the study were carried out off-line and aliquots of the reaction mixtures were spotted on the DIOS target. Therefore, sample deposition speed and precision becomes the most crucial point for the application of this system. By employing an electrospray deposition device [66], the sample homogeneity could be significantly improved, thus leading to better results in the quantification and a much higher reproducibility. This approach clearly demonstrates the high potential of the DIOS-MS-based assay scheme to be used in a high-throughput screening for either enzymatic activities or potential inhibitors.

2.3.3 SAMDI-MS-based assay schemes

The group of Mrksich and co-workers developed a MALDI-based assay scheme employing a target surface modification by self-assembled monolayers (SAM) [67]. The SAMs used were designed to present a mixture of oligo(ethylene glycol) groups and substrates, e.g., peptides or carbohydrates, as terminal groups. The oligo(ethylene glycol) is crucial, since it prevents non-specific interactions of proteins with the surface, ensuring that all interactions of proteins in solution occur with the immobilized substrates. To ensure a maximum of accessibility to the immobilized substrates, SAMs presenting the substrate and oligo(ethylene glycol) terminal groups in a ratio

of 1:4 are created. The use of these functionalized monolayers in monitoring enzymatic activity was tested using β-1,4-galactosyltransferase as model enzyme and immobilized *N*-acetylglucosamine as substrate. The enzyme solution was incubated on the SAM-modified target surfaces. After a certain incubation time, the target is rinsed, matrix is applied and MALDI-MS analysis can be performed. By varying the incubation times, time-resolved reaction profiles could be generated, allowing to obtain kinetic information about the enzyme-catalyzed reaction. The yield of the enzymatic conversion was calculated from the ratio of product signal to the sum of product and substrate signal intensities (see above). This so-called SAMDI (*s*elf-*a*ssembled *m*onolayers for MAL*DI*) assay scheme was used in several other studies from the same group.

An approach to multiplexed analysis was presented by Min and colleagues [68]. They developed a SAMDI-based assay scheme for the activity screening of different kinases. In this assay scheme, peptide substrates were employed, which are specific for a certain type of kinase. A mixture of these substrates (in this case of four) was immobilized on the SAM. After incubation with an appropriate kinase, the target surface was rinsed, thus stopping the reaction, matrix was deposited on the surface and MALDI-MS analysis was performed. Enzymatic activity was determined by monitoring the signal intensities for the substrates and the products formed, demonstrating the feasibility of the SAMDI approach for multiplexed analysis of enzymatic activities. In a second set of experiments, the possibility of quantification of enzyme inhibition by means of SAMDI-MS was studied. Therefore, two different kinases were

incubated separately with varying amounts of known inhibitors on the modified target surface. The yield of the respective reactions in the presence of the inhibitors was determined by MALDI-MS and IC_{50} values for both compounds could be generated, indicating the general applicability of the SAMDI-MS approach for quantifying enzyme inhibition. However, the reported IC_{50}-value for the inhibition of casein kinase I was significantly higher than the value derived from a different assay scheme [69]. This might be contributed to the differences between a liquid-phase reaction and a surface-liquid-phase reaction or the general problem of accurate quantification of the analytes in MALDI.

In another paper, Min et al. report the development of a SAMDI-MS based screening procedure for the identification of anthrax lethal factor inhibitors [70]. Anthrax lethal factor is a zinc-dependent protease. As model substrate, an oligopeptide, which is cleaved by the enzyme at a proline position, was immobilized on the SAM-modified surface of a MALDI target. The target plate was created to offer an array of 100 gold-coated and SAM-modified sample spots. For the first screening, mixtures of eight potential inhibitors each were added to an aliquot of the enzyme solution. These mixtures were then incubated on the target sample spots. After a defined time, the complete target plate was rinsed, thus stopping the enzyme reactions in each well, matrix was added and MALDI-MS analysis was performed. Inhibition activity was assessed, when no or only small product signal intensity was observed. After this pre-screening, the compounds present in the wells, where complete or partial inhibition was determined, were screened individually. Following this

procedure, one compound could be identified, which completely inhibited the enzymatic activity of anthrax lethal factor. Incubating the enzyme in presence of varying amounts of inhibitor and determining the relative amount of product formed during the reactions allowed the quantification of the inhibition activity. From these assays, an IC_{50}-value for the inhibitor was calculated, that fitted well to the value obtained by a UV-absorption based solution-phase assay. This study clearly shows the high potential of the SAMDI-MS approach for being employed in chemical screenings for inhibitory activity in a high-throughput environment such as drug discovery. Using a variation of the SAMDI-MS approach for monitoring enzymatic conversions, Min et al. developed a so-called pull down assay scheme (see figure 2.6).

In this assay scheme, the enzymatic reaction is carried out completely in solution phase. Aliquots of the reaction mixture are then transferred to a SAM-modified MALDI target, where the remaining substrate and the reaction product are selectively immobilized. After extraction of the analytes, the target is rinsed, treated with matrix, and MALDI-MS analysis is performed. The major advantage of this assay scheme is that the inherent danger of negative influences on the reaction kinetics by immobilization of the substrate as in standard SAMDI-MS-based assay formats is circumvented. Furthermore, by selectively extracting the analytes of interest and removal of the other reaction mixture constituents, the chemical background during the MS analysis can be significantly reduced. The described pull down SAMDI assay format was demonstrated to yield quantitative information about enzyme-catalyzed reactions for the example of the protein arginine methyltransferase 1-mediated methylation of a peptide substrate [71].

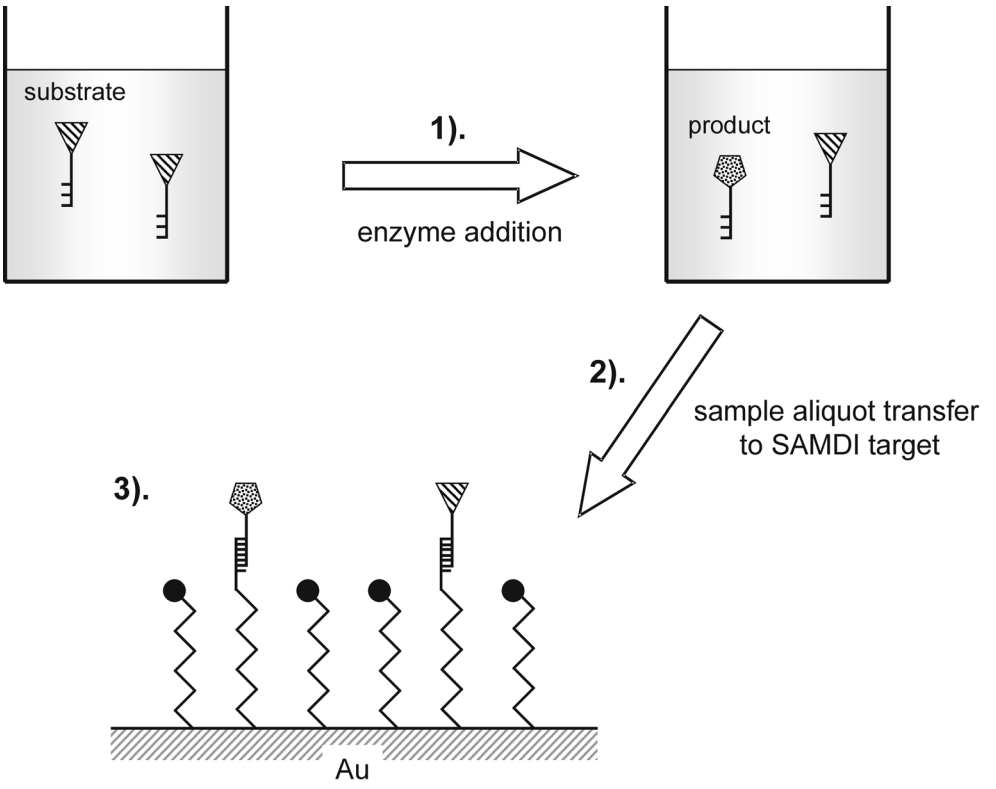

Figure 2.6: Scheme of a SAMDI-MS-based pull down assay. Substrate conversion is initiated by addition of the enzyme (1); sample aliquots are removed at several time points from the reaction mixture and transferred to the SAM-modified MALDI target (2); analytes (product and remaining substrate) are immobilized selectively on the SAM by binding to incorporated functionalities (3) [71].

2.3.4 MALDI-MES assay schemes

Schlüter and co-workers developed an enzyme screening assay for the fast determination of enzymatic activities of protein fractions [72, 73]. The MALDI-MES (mass-spectrometry-assisted enzyme screening) system is based on the idea of immobilizing enzymes present in protein mixtures on affinity beads and to subsequently screen for certain activities of these enzymes. Therefore, substrates have to be selected, which are specific for a target enzymatic activity. The general MALDI-MES approach is a five-step procedure.

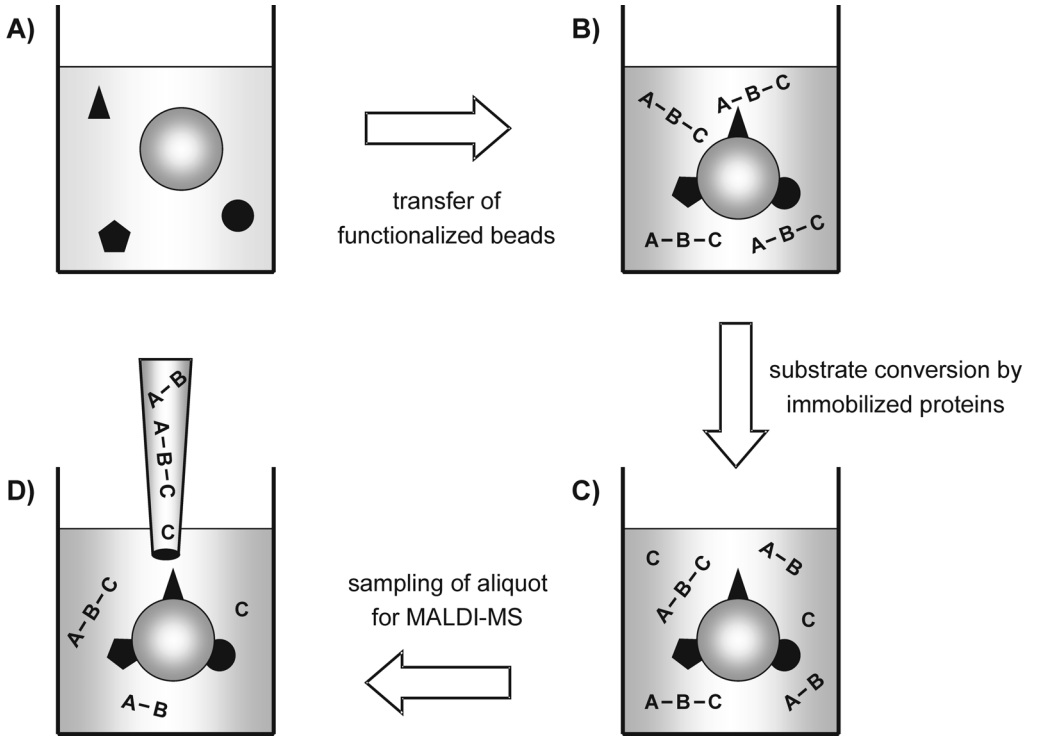

Figure 2.7: Scheme of MALDI-MES assay procedure. Activated sepharose beads are incubated with an enzyme mixture, thus immobilizing the enzymes on the beads (A); after rinsing, the cleaned beads are transferred to the substrate solution and the reaction is started (B); substrate conversion is taking place mediated by the immobilized enzymes (C); sample aliquots are taken from the reaction mixture and transferred to a MALDI target; beads are remaining in the assay solution (D);

In the first step, cyanogen bromide-activated sepharose beads are added to a protein fraction from a natural product extract. The enzymes present in the extract are non-specifically covalently bound to the beads. The beads are cleaned by several rinsing steps, thus removing all matrix components, which are present in the extract. The functionalized beads are transferred in the second step to a reaction vial containing a target-specific substrate probe dissolved in pure water. In the third step, the beads are incubated with the substrate and product formation can take place, provided that the target

enzyme is present on the bead. Aliquots are taken from the reaction mixture at different times and deposited on a MALDI target (fourth step). The last step of the procedure is the MALDI-MS analysis of the sample spots and the relative quantification of the product and substrate concentration in each sample. This allows to obtain time-resolved reaction profiles, which can be evaluated to assess the activities of the enzymes immobilized on the beads. The major advantage of the MALDI-MES assay scheme is the extraction of the enzymes from the crude sample. This allows the enzymatic reaction to be performed in a clean environment without the presence of compounds, which might interfere with the subsequent MALDI-MS analysis of the reaction mixture, such as salts, proteins or peptides present in the extract. Due to the immobilization of the enzymes on the affinity beads, even disturbances of the enzymes themselves in the MS analysis are prevented. Furthermore, the autoproteolysis of the enzymes is hindered by the immobilization, thus stabilizing the sample. The MALDI-MES approach was successfully applied by Schlüter et al. to the determination of enzymatic activities in renal tissue extracts. They were also able to perform inhibition studies, by adding the enzyme-functionalized beads to substrate solutions containing an inhibitor. However, it must be mentioned that the quantification of the substrate and product components was found to be not very accurate, even when using an internal standard. Therefore, this method remains semi-quantitative. On the other hand, the accuracy of quantification is always high enough to allow a reliable assessment of enzymatic activity by determining the ratio of product and substrate signals [73].

2.3.5 Pre-steady-state kinetics by MALDI-MS

Despite the fact that exact quantification of analytes by means of MALDI-MS is considered to be problematic, Houston et al. presented in 2000 the first approach of using MALDI-MS for studying the pre-steady-state kinetics of an enzymatic conversion [74]. As model system, they studied the dephosphorylation reaction catalyzed by phosphatase Stp1, which was isolated from *Schizosaccharomyces pombe*. In order to obtain the rate constants for the individual mechanistic steps, they monitored the formation of the phosphorylated enzyme complex in the pre-steady-state regime of the conversion. Samples were generated using a rapid-mix quench-flow instrument as depicted in figure 2.8.

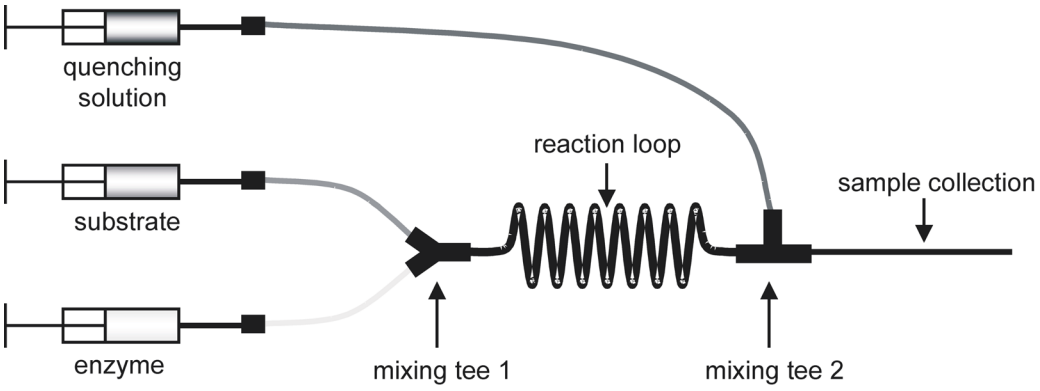

Figure 2.8: Schematic set-up of the rapid-mix quench-flow device for off-line pre-steady-state kinetic measurements by MALDI-MS. One syringe provides a constant stream of enzyme solution, the other of substrate solution. The reaction is initiated by rapid mixing in mixing tee 1. The reaction is stopped upon mixing with the quenching solution provided by the third syringe in mixing tee 2. Variation of reaction times is achieved by changing the length of the reaction loop or the flow rates of the pumps. The quenched reaction solution is collected, mixed with matrix and analyzed by means of MALDI-MS [74].

The set-up comprises three syringe pumps, which deliver enzyme, substrate and quenching solutions. The reaction is initiated by rapid mixing of enzyme and substrate solutions in the first mixing tee. The reaction is terminated by quenching the mixture with a 0.3% trifluoroacetic acid solution in the second mixing tee. Different reaction times are realized by varying the length of the reaction capillary or the reactant solution flow rates. The quenched samples were collected, mixed with a matrix, deposited on a target and analyzed by MALDI-MS. With this set-up, the concentration of the target intermediate species could be determined at reaction times between 4 ms and 1 s. Quantification was performed using the ratio of analyte signal intensity to the sum of signal intensities of the analyte and the non-phosphorylated, native enzyme present at each time point (see above). The reaction progress curves obtained by the MALDI-MS experiments were compared to those derived from stopped-flow UV-absorption-based measurements and were found to be in good agreement. The authors used the set-up to study the effect of employing various different compounds as substrate in the reaction, finding significant differences in the kinetic behavior of the enzyme.

Gross and colleagues used a similar experimental set-up in order to study the pre-steady-state kinetics of reactions catalyzed by dTDP-glucose 4,6-dehydratase. They were able to identify a relevant reaction intermediate and by following its build up and decrease during the reaction, rate constants for most reaction steps could be determined. With this study, the authors could validate the proposed mechanism for this enzymatic reaction [75].

These experiments demonstrate the general applicability of MALDI-MS in combination with a rapid-mix quench-flow set-up to investigate the pre-steady-state kinetics of enzymatic conversions, with the possibility to directly identify and monitor the formation of key reaction intermediates.

2.4 Conclusions

With the high demand for rapid, reliable means of assessing enzymatic activities and enzyme inhibition, mass spectrometry offers an exciting alternative to the established assay schemes based on fluorescence, UV/vis-absorption or radioactivity. Due to the increasing interest in this fascinating technique, MS-based assay schemes are today widely employed in the areas of enzymology, pharmaceutical and clinical chemistry.

By offering the possibility to use naturally occurring substrates, MS-assays allow direct insight into biological relevant catalytic mechanisms. Kinetic data about an enzymatic conversion can be obtained from the steady-state regime of a reaction as well as from its pre-steady-state. Furthermore, MS-based detection allows the direct identification of reaction intermediates and unexpected side-products. Another advantage is the versatility of the MS-based detection, which enables the rapid screening of large numbers of different compounds for potential use as enzyme substrates without the need for laborious synthesis of spectroscopically detectable compounds. The suitability of MS detection method for multiplexed analysis, assaying several different substrates with one enzyme in a single experiment, offers a range of

new possibilities in the bioanalytical chemistry. This “functional proteomics” methodology allows to either identify certain species by their characteristic activity pattern or classify unknown enzymes by their respective functionalities. Another possibility would be the screening for alternative substrates of known enzymes, thus allowing a fast assessment of the possible use of enzymes as biocatalysts in the production of novel chemicals. In addition to the determination of enzymatic activity, inhibition studies can be performed very easily.

Both, ESI- and MALDI-MS offer interesting features for their application in monitoring of enzymatic bioassays. MALDI-based assay schemes are predestined to be employed in array assay formats, which can be used for high-throughput applications. However, quantification remains problematic in MALDI-MS. Several approaches were undertaken to increase the quality of data. Nevertheless, MALDI-MS offers sufficient accuracy and reproducibility to allow at least the semi-quantitative determination of enzymatic activities in general activity or inhibition screening procedures. Another critical aspect is the deposition of samples on the MALDI target plate. Pipetting samples onto the target frequently results in inhomogeneous distribution of the analytes in the matrix, thus leading to poor reproducibility. Electrospray deposition provides an interesting alternative for sample deposition, resulting in a much better sample homogeneity. The speed of sample deposition, however, remains to be a challenge. While several groups reported extremely short analysis times for a complete array, target preparation and sample deposition may still be comparably time-consuming.

ESI-MS-based assay schemes have the advantage of being directly compatible to the liquid-phase reaction conditions. Therefore, it is possible to monitor enzymatic conversions on-line by coupling the reaction vessel directly to the ionization interface of the mass spectrometer. Additionally, the accuracy of quantification and sample-to-sample reproducibility are usually better, compared with MALDI-MS schemes. However, the sample throughput capability of ESI-MS-based assays is limited. With the use of a fast autosampler, it is possible to reach analysis times of approximately one minute per sample. This rate should allow ESI-MS assay formats to be employed in at least medium throughput environments.

A general challenge when using MS-based detection is the sample preparation procedure. Particularly, ESI-MS analyses are prone to be deteriorated by the presence of matrix components such as salts, involatile buffers and biomolecules. However, despite its relatively high tolerance against matrix effects, extreme amounts of matrix background can also decrease the sensitivity of MALDI-MS significantly. Therefore, sample clean-up procedures should be taken into account, when analyzing directly from a reaction mixture. Approaches to this have been made by selectively extracting the analytes from the reaction mixture, followed by a rinsing step to remove the disturbing compounds. These schemes have been developed for both ESI- and MALDI-based assays, but are particularly useful for MALDI-MS, where the analytes can be trapped directly on the target, rinsed and subsequently analyzed. However, these methods require, when applied in

ESI-MS assay schemes, a time-consuming chromatographic step. Therefore, a faster means of effective sample clean-up might be desirable.

Generally, the application of mass spectrometry for detection in enzymatic bioassays offers very intriguing possibilities. MS-based assay schemes certainly cannot replace the optical or radioactivity-based assay schemes in routine analysis, but they are an extremely valuable alternative providing access to areas of enzymology, which could not be studied by the established methodologies. Furthermore, MS-detection schemes seem to have a great potential for the application in high-throughput screening procedures in drug development and biocatalysis research.

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Chapter 3

Strategies to Employ ESI-MS for the Monitoring of Enzymatic Reactions[‡]

Different strategies for employing electrospray ionization mass spectrometry (ESI-MS) for the monitoring of enzymatic conversions are presented: the at-line approach, which provides time-resolved reaction profiles, the on-line approach, which allows the rapid assessment of enzymatic activities and the on-chip approach, which can be employed in the miniaturized monitoring of enzyme-catalyzed reactions.

The general feasibility of the ESI-MS detection approach was demonstrated by comparing the ESI-MS approach with an independent assay technique, the enzyme-amplified lanthanide luminescence (EALL) assay, as a reference method. The assay performances were found to be in good agreement with each other, thus validating the ESI-MS approach. As model reaction, the esterase-catalyzed cleavage of bis(2-pyridylmethyl)(2-acetoxyphenyl)amine was selected.

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3.1 Introduction

A widely used detection method for the monitoring of enzymatic conversions is fluorescence spectroscopy due to its low limits of detection. In 1991, Gudgin Templeton et al. introduced the principle of enzyme-amplified lanthanide luminescence (EALL) as a further development of the fluorescence-based detection [1]. This technique, which is a highly selective and sensitive method of detection in bioassays, is based on the luminescence of a selection of rare earth ions. It is known that of all rare earth metal ions only four, namely samarium(III), europium(III), terbium(III) and dysprosium(III), may emit light in the visible part of the electromagnetic spectrum. However, it is hard to evoke this luminescence in a satisfactory way, because the naked metal ions do not absorb enough of the excitation energy [2]. It is possible to circumvent this problem by complexing the lanthanide ions with certain organic molecules, which subsequently act as ‘sensitizers’, if the energy level of the ligand matches that of the lanthanide ion. This means that the organic molecule transfers absorbed energy onto the lanthanide ion, which then shows emission at its characteristic wavelength. Due to this energy transfer and an intersystem crossing in the ligand molecule, the luminescence is extremely long lived so that time-resolved measurements are possible. Additionally, those complexes show very large Stokes’ shifts often exceeding 250 nm, allowing for time-resolved lanthanide luminescence measurements with extreme selectivity and sensitivity. The second reason for the high performance of the EALL technique is an enzymatic amplification reaction, which is performed previously to the luminescence measurements. In this

enzymatic reaction the ligand, which later on acts as sensitizer for the lanthanide ion, is formed.

In their above mentioned article, Gudgin Templeton et al. describe the detection of the enzymes alkaline phosphatase, xanthine oxidase, β-galactosidase and glucose oxidase with terbium(III) and different salicylates, which act here as sensitizers [1]. Based on the EALL method for alkaline phosphatase, a number of assays for various biomolecules were developed in the following years [3-6].

Further work was focused on the development of new EALL methods for other hydrolytic enzymes. A new sensitizer for terbium(III) was discovered by screening a small library of tripod ligands [7]. The acetic acid ester of the sensitizer was synthesized, and its hydrolysis by porcine liver esterase under release of the sensitizer was determined by time-resolved fluorescence spectroscopy after complexation with terbium(III). An excitation wavelength of 290 nm and an emission wavelength of 545 nm were applied for this purpose. Limits of detection for porcine liver esterase were 10^{-9} mol L^{-1}. The ligand itself is non-fluorescent, but able to transfer excitation energy to Tb(III), the characteristic emission of which is subsequently observed. Thus, the reaction can be monitored by means of lanthanide luminescence.

A viable alternative for the monitoring of this enzymatic conversion is offered by the ESI-MS-based detection approach. Therefore, different strategies of employing ESI-MS as means of detection were explored. The general suitability of the MS assay scheme was investigated by comparing experiments based on the EALL methodology.

3.2 Experimental

Chemicals

Bis(2-pyridylmethyl)(2-hydroxybenzyl)amine was kindly provided by Dr. Florian Schweppe and Prof. Dr. Bernt Krebs (Institute for Inorganic and Analytical Chemistry, University of Münster/Germany). Bis(2-pyridylmethyl)-(2-acetoxyphenyl)amine was synthesized according to literature [7]. Bis-tris-propane and esterase from porcine liver were purchased from Sigma (Deisenhofen/Germany) in the highest purity available. Terbium(III) chloride hexahydrate was from Aldrich (Steinheim/Germany) and dimethylsulfoxide (p.a.) was purchased from Merck (Darmstadt/Germany). Solvents for LC were methanol (gradient grade) and water in gradient grade quality from Acros (Geel/Belgium) and in LC-MS grade quality from Biosolve (Valkenswaard/The Netherlands).

Instrumentation

All fluorimetric determinations were carried out with the microplate reader model FLUOstar from BMG LabTechnologies (Offenburg/Germany) with FLUOstar software version 2.10-0. Due to the fact that no filter with the exact excitation wavelength of the complex (297 nm) was available, excitation was accomplished at a wavelength of 290 nm (± 15 nm). For emission measurements, a dedicated narrow-bandwidth filter for Tb(III) emission at λ = 545 nm (± 10 nm) was used.

Electrospray ionization-mass spectrometry (ESI-MS) detection of the “at-line” assays was performed on a Shimadzu LCMS QP8000 single quadrupole

mass spectrometer with an electrospray ionization probe coupled to a flow-injection system comprising a SIL-10A autosampler, a SCL-10Avp controller unit and two LC-10ADvp HPLC pumps (all from Shimadzu, Duisburg/Germany). The setup control and data evaluation were performed using the Shimadzu Class 8000 V1.20 software.

The “on-line” experiments were performed using a flow-injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/Germany) and a 74900 series infusion pump (Cole-Parmer, London/UK).

For the “on-chip” experiments, two syringe infusion pumps were used. The chip reactor was kindly provided by Dr. Monica Brivio, Dr. Wim Verboom and Prof. Dr. David N. Reinhoudt, Department of Supramolecular Chemistry and Technology and MESA$^+$ Institute for Nanotechnology, University of Twente/The Netherlands). Fabrication and chip parameters are described in [8].

For detection in the “on-line” and the “on-chip” experiments, an Esquire 3000$^+$ ion trap mass spectrometer (Bruker Daltonik, Bremen/Germany) equipped with a standard ESI source was used. The resulting data were analyzed using DataAnalysis software version 3.1 (Bruker Daltonik, Bremen/Germany).

Solution preparation

The esterase from porcine liver as well as the terbium chloride solution, the bis-tris-propane buffer and the acetic acid ester of HL_1 were dissolved in water. To ensure sufficient solubility of the ester, 20 % (v/v) dimethylsulfoxide were added to the stock solution with the concentration of $1*10^{-3}$ mol/L. All further dilution steps were made by addition of water to the stock solution.

Time-resolved fluorescence measurements (on-line)

50 μL of a $1*10^{-3}$ mol/L Tb(III) solution were pipetted in 3 × 12 wells of a 96 well microplate. Afterwards, 50 μL of ester solution with the concentrations $1*10^{-4}$ mol/L, $6*10^{-5}$ mol/L and $3*10^{-5}$ mol/L were added in 12 wells each. Then, 50 μL of bis-tris-propane buffer solution (pH 7.4, 0.01 mol/L) were added into each well. In the last 4 wells of each row, 50 μL of water were pipetted for the blank and in the remaining 3 × 8 wells 50 μL of esterase solution (1 u/mL) were transferred. Afterwards, the fluorescence intensity was measured with a delay time of 50 μs and an integration time of 1 ms. Excitation wavelength was 290 nm and emission wavelength was 545 nm. The fluorescence intensity was determined in intervals of five minutes.

Time-resolved fluorescence measurements (off-line)

For all three concentrations of ester ($1*10^{-4}$ mol/L, $6*10^{-5}$ mol/L and $3*10^{-5}$ mol/L) the measuring procedure was as follows: First, 5 mL of ester solution and 5 mL of esterase solution (1 u/mL) were mixed. 100 μL of this mixture were pipetted immediately in twelve wells of different microplates each. Another row of those plates was filled with 50 μL of ester solution and

50 μL water. Every five minutes, 50 μL of terbium chloride solution and 50 μL of buffer were added to one of the plates. Subsequent fluorescence measurements of this plate were accomplished as for the on-line measurements.

Mass spectrometric "at-line" approach

For all three concentrations of ester ($1*10^{-4}$ mol/L, $6*10^{-5}$ mol/L and $3*10^{-5}$ mol/L), the mass spectrometric at-line procedure was as follows: 2 mL of esterase solution (1 u/mL) and 2 mL of ester solution were combined and mixed. Of this reaction mixture, 1.5 mL were transferred into a HPLC vial, from where an autosampler injected first after 2.25 minutes reaction time 5 μL of the reacting solution into a solvent stream consisting of methanol/water (50/50, v/v) with a flow rate of 0.2 mL/min. Subsequent injections out of the reaction vial took place every 4 minutes. Thus, the samples were introduced into the ESI mass spectrometer. They were analyzed in the positive ion mode using the selected ion monitoring (SIM) mode. The signal traces were set to *m/z* 328, 306, 370 and 348 for the protonated ester, the resulting phenol and their sodium adducts, respectively. For all measurements, a curved desolvation line (CDL)-voltage of -35 V and a CDL temperature of 230 °C were used. The deflector voltage was 35 V and the detector voltage was 1.6 kV. The ESI parameters comprised a probe voltage of +3.0 kV and a nebulizer gas flow-rate of 4.5 L/min.

Mass spectrometric "on-line" approach

Esterase solutions of varying concentration (0.05 mg/L, 0.07 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L and 0.7 mg/L) were prepared and 10 μL of each solution were injected six-fold into the FIA system. The carrier stream was set to 0.1 mL/min of pure water. The substrate solution (c(ester) = $6*10^{-5}$ mol/L) was infused by a syringe pump at a rate of 10 μL/min. All mass spectra were measured in the positive ion full scan mode employing the ESI source with the instrumental settings as shown in table 3.1.

Table 3.1: List of ESI-MS parameters for Esquire 3000^{+}

Parameter	Settings
nebulizer gas (N_2) pressure	30.0 psi [a] / 5.0 psi [b]
dry gas (N_2) flow	7.0 L/min [a] / 4.0 L/min [b]
dry gas (N_2) temperature	350 °C [a] / 250 °C [b]
capillary high voltage	4639 V
Capillary exit voltage	117.1 V
skimmer voltage	40.0 V
octopole 1 voltage	12.0 V
octopole 2 voltage	1.7 V
octopole amplitude	120.0 Vpp
lens 1 voltage	-5.0 V
lens 2 voltage	-60.0 V
trap drive level	47.9

[a] on-line experiments; [b] on-chip experiments

Mass spectrometric "on-chip" approach

The on-chip experiments were carried out using a borosilicate chip-based micro-reactor with a 197 mm long, 200 μm wide and 100 μm deep reaction channel (reaction volume = 4 μL). The esterase solution (1 u/mL) and the

substrate solution ($6*10^{-5}$ mol/L) were fed into the micro-reactor using two syringe pumps. Different reaction times were realized by varying the flow rates of the reactand solutions. The detection was carried out in the positive ion mode measuring full scan spectra with the ESI-MS parameters settings as given in table 3.1.

3.3 Results and discussion

3.3.1 EALL reference method

The esterase-catalyzed hydrolysis of bis(2-pyridylmethyl)(2-acetoxyphenyl)-amine is presented in figure 3.1.

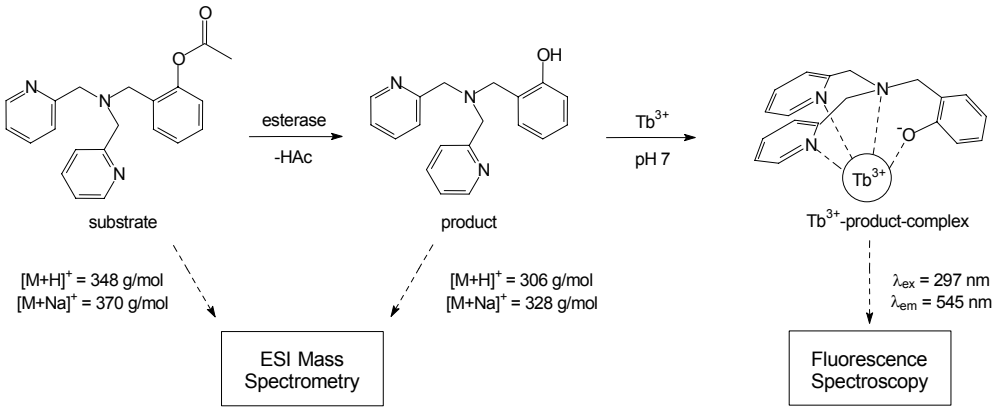

Figure 3.1: Reaction scheme for the enzymatic cleavage of the ester of HL_1 and the complex formation of the product with Tb(III) as well as detection scheme for the fluorescence spectroscopic and ESI-mass spectrometric determination of substrate and product.

In contrast to the substrate, the reaction product bis(2-pyridylmethyl)-(2-hydroxybenzyl)amine (HL_1) fulfils both requirements for lanthanide-sensitized luminescence: It forms a complex with Tb(III) and is able to transfer its excitation energy to the central ion, resulting in intense luminescence at

λ = 545 nm. The lanthanide luminescence may be detected in the time-resolved mode with a delay time of 50 μs and an integration time of 1 ms. In contrast to other lanthanide luminescence systems, substrate and product molecule are non-fluorescent and are characterized by very similar UV/vis absorption spectra. Therefore, both native fluorescence spectroscopy and UV/vis spectroscopy are not suitable for reaction monitoring of this enzymatic conversion. This is illustrated in figure 3.2, where the UV/vis spectra of approximately $1*10^{-4}$ mol/L solutions of substrate and product are presented.

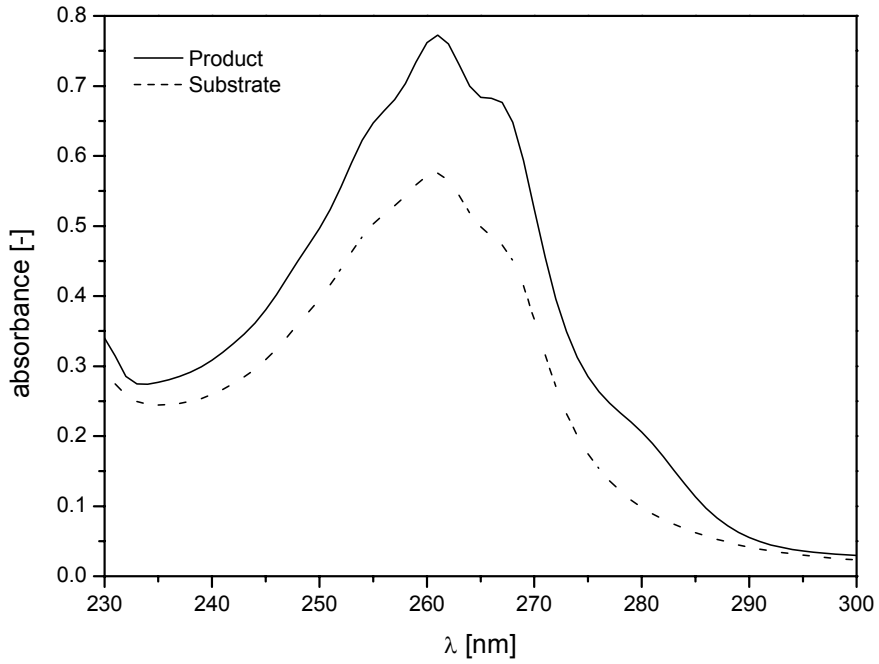

Figure 3.2: UV/vis-absorption spectra of substrate (HL_1E) and product (HL_1).

Both exhibit significant absorbance in the wavelength range between 230 nm and 300 nm. However, as the absorbance for both substances can be traced back mostly on the three aromatic rings, and only to a lesser extent on the additional functional groups, the differences between the UV/vis spectra of

both substances are so small that they cannot be used to monitor the enzymatic conversion.

Initially, the suitability of this system for a true on-line monitoring of the enzymatic conversion was investigated. Depending on the substrate concentration, the maximum fluorescence was measured between 10 and 20 minutes. However, the fluorescence intensity decayed rapidly again, and no constant value was obtained. As the same wells of the microplate are irradiated multiply with UV light below 300 nm when using this approach, it is assumed that photobleaching of the substrate and/or product molecules occurs. For this reason, an off-line approach was developed. Here, any well of the microplate is read out only once, reducing the likelyhood of photobleaching. For the off-line measurements, constant fluorescence intensity was observed after 40 minutes for all three substrate concentrations investigated. The respective data are presented in Figure 3.3.

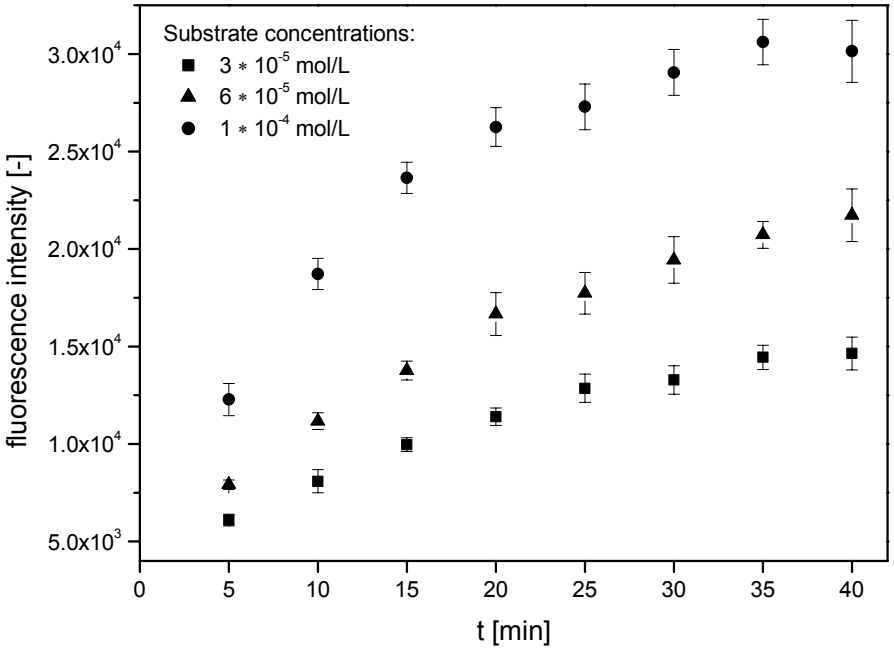

Figure 3.3: Reaction monitoring of the ester cleavage by time-resolved fluorescence spectroscopy.

Calibration was performed externally using pure standards of substrate and product. The error bars represent the relative standard deviation (n = 8) for multiple determination of the fluorescence intensity in different wells of one microplate. Under the conditions applied in this work, the limit of detection for the product is $1*10^{-5}$ mol/L.

3.3.2 ESI-MS "at-line" approach

Using the above described EALL method as a reference, alternative MS-based methods for the monitoring of the enzymatic conversion were developed. Due to the basic properties of both substrate and product, both are well suited for being detected by means of MS. The substrate was detected at the signal trace of m/z 348 for the protonated molecule and at m/z 370 for its sodium adduct. For the product, m/z 306 and m/z 328 were selected to monitor the protonated molecule and the sodium adduct, respectively.

The instrumental set-up of the at-line approach was as follows:

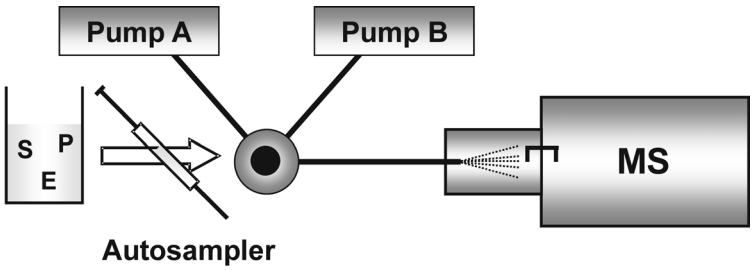

Figure 3.4: Schematic set-up of the at-line approach comprising a FIA-system with two HPLC pumps and an autosampler coupled directly to the ESI mass spectrometer.

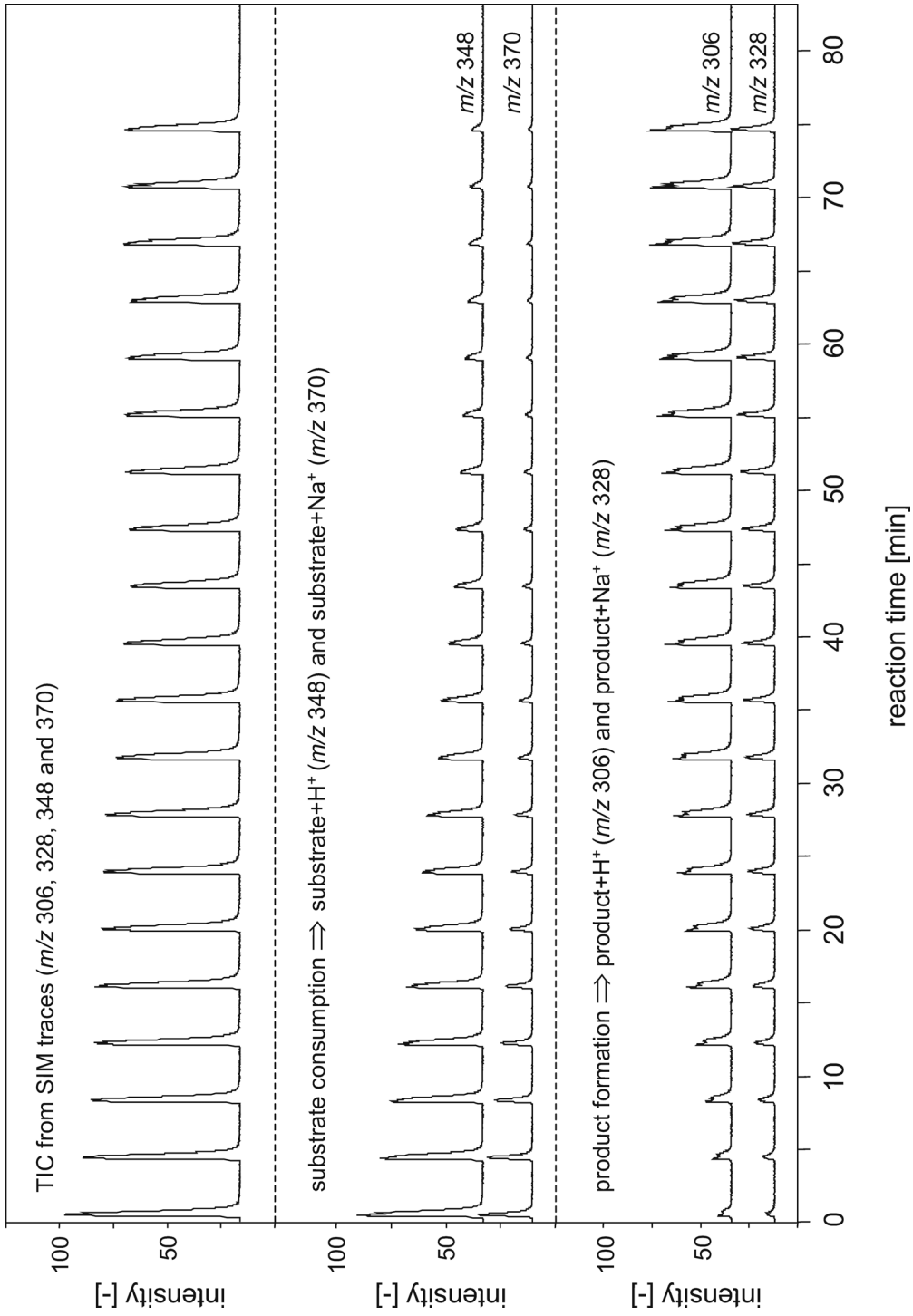

Figure 3.5: SIM total ion current and individual SIM mass traces for the proton and sodium adducts of substrate and product.

The reaction was started by combining the esterase and substrate solutions in a HPLC-vial. The reaction vessel was placed into the autosampler of the FIA system and 5 μL aliquots of the active reaction mixture were injected into the mass spectrometer every four minutes. Monitoring the product and the substrate at their respective signal traces resulted in the following injection profile. In the upper part of figure 3.5, the total ion current (TIC) of the sum of the selected ion monitoring (SIM) traces of m/z 306, 328, 348 and 370 is presented. As the TIC is decreasing slightly with time, it is obvious that the two ions associated with the substrate are detected with slightly better intensity than the two respective ions of the product. The individual signal traces are presented in the lower parts of figure 3.5.

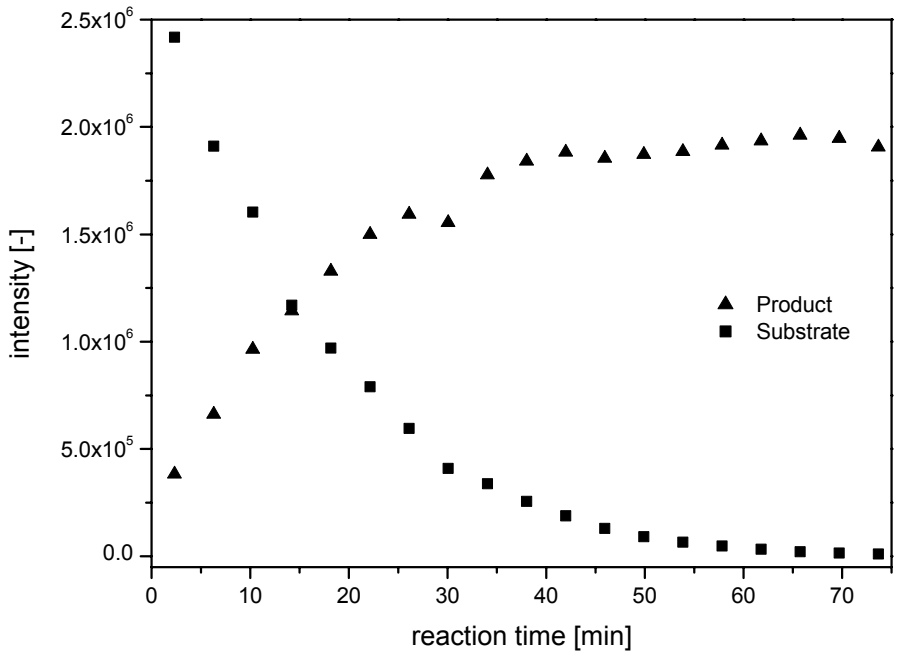

Figure 3.6: Time-resolved reaction profile of the ester cleavage by at-line ESI-MS. The $[M + H]^+$ and $[M + Na]^+$ peaks have been summed up for substrate and product.

In Figure 3.6, the sum of the intensity of the $[M + H]^+$ and $[M + Na]^+$ peaks for substrate and product is presented depending on the reaction time.

It is obvious that, as in case of the fluorescence measurements, a constant value is observed already after 40 minutes for a concentration of $6*10^{-5}$ mol/L. For the other substrate concentrations, the data from time-resolved fluorescence spectroscopy were confirmed as well. Under these conditions, the limits of detection for the substrate and the product are $1*10^{-5}$ mol/L, respectively, which is the same as for the reference fluorescence method.

3.3.3 ESI-MS "on-line" approach

As a second approach to employ ESI-MS as means of detection in enzymatic bioassays, a true on-line reaction system was set up.

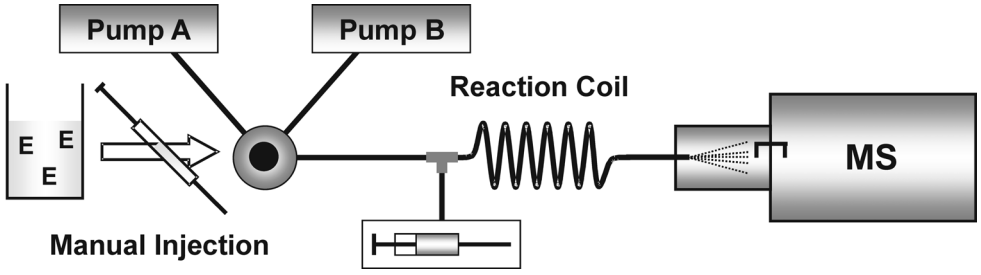

Figure 3.7: Schematic set-up of the on-line approach comprising a FIA-system with two HPLC pumps and a manual injection valve, a syringe pump and a 25 m teflon reaction coil coupled to the ESI mass spectrometer.

The syringe pump, which delivered a constant stream of the substrate mix solution, was connected via a mixing tee to the flow path. Downstream of the mixing tee, a reaction coil was inserted to give a delay time of approximately

three minutes. In this system, the enzyme solutions were injected manually several time by means of a biocompatible injection valve.

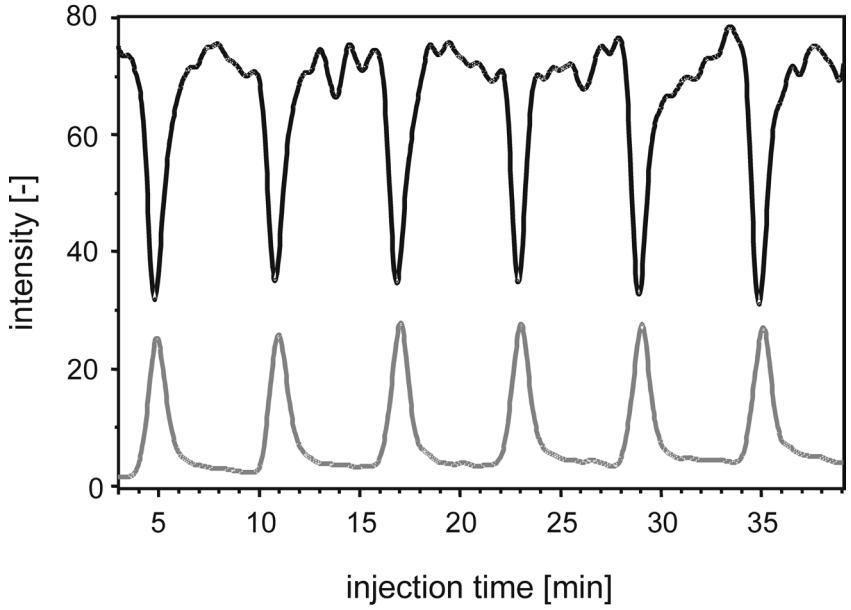

Figure 3.8: Signal development for substrate and product at six-fold injection of esterase solution (c(esterase) = 0.2 mg/L).

When the enzyme solution plug reaches the mixing tee, the reaction starts and the substrate is converted into the product. The reaction is stopped upon nebulization of the eluent in the ESI interface. The length (volume) of the reaction capillary and the flow rates of the carrier stream and the substrate solution were kept constant throughout the experiments. Therefore the reaction time is always the same. The product and substrate signal traces are continuously monitored by the ESI mass spectrometer. As soon as the reaction plug reaches the detector, a drop of signal intensity for the substrate and increasing signal intensity for the product is observed. The area underlying the peaks is directly proportional to the enzyme concentration. Therefore, the on-line approach may be used for the quantification of enzyme

content. The lower limit of detection was found to be 0.07 mg/L at signal-to-noise value ≥ 10 and a linear range up to an esterase concentration of 0.7 mg/L with a linear regression factor of 0.999. The limits of detection are not really competitive in comparison with other methodologies, but the feasibility of the on-line approach for the assessment of enzymatic activities could be proven.

3.3.4 ESI-MS "on-chip" approach

Due to the often limited amounts available for many samples and the high costs of reagents, there is a sound demand for miniaturization in modern bioanalytical chemistry. Although the sample and reagent consumption in the described ESI-MS assays is already comparably low, further miniaturization is possible by employing chip-based micro-reactors. Therefore, an on-chip assay system with mass spectrometric detection was developed.

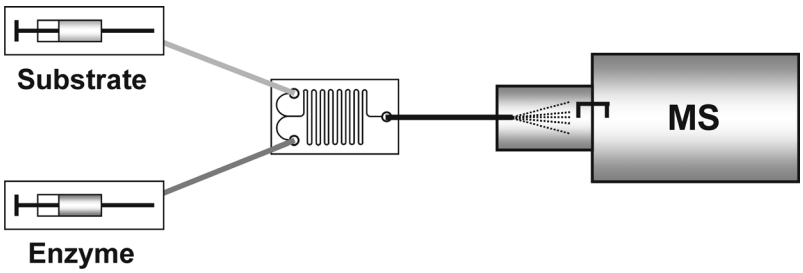

Figure 3.9: Schematic set-up of the on-chip assay system comprising two syringe pumps for delivery of constant streams of enzyme and substrate solution coupled to the inlets of a chip-based micro-reactor. The outlet of the reactor is directly coupled to the ESI mass spectrometer.

Constant streams of both, the enzyme and the substrate solution are provided by two syringe pumps. Mixing of the two reactands and the conversion take place on the chip. The reaction is stopped by the nebulization of the reaction

mixture in the ESI interface. Due to the fixed reaction volume (as defined by the chip reactor and the transfer capillary to the mass spectrometer), the reaction time depends only on the total flow rate (combined enzyme and substrate streams) applied. At a given flow rate, the reaction can be monitored in the detector in a “steady-state”.

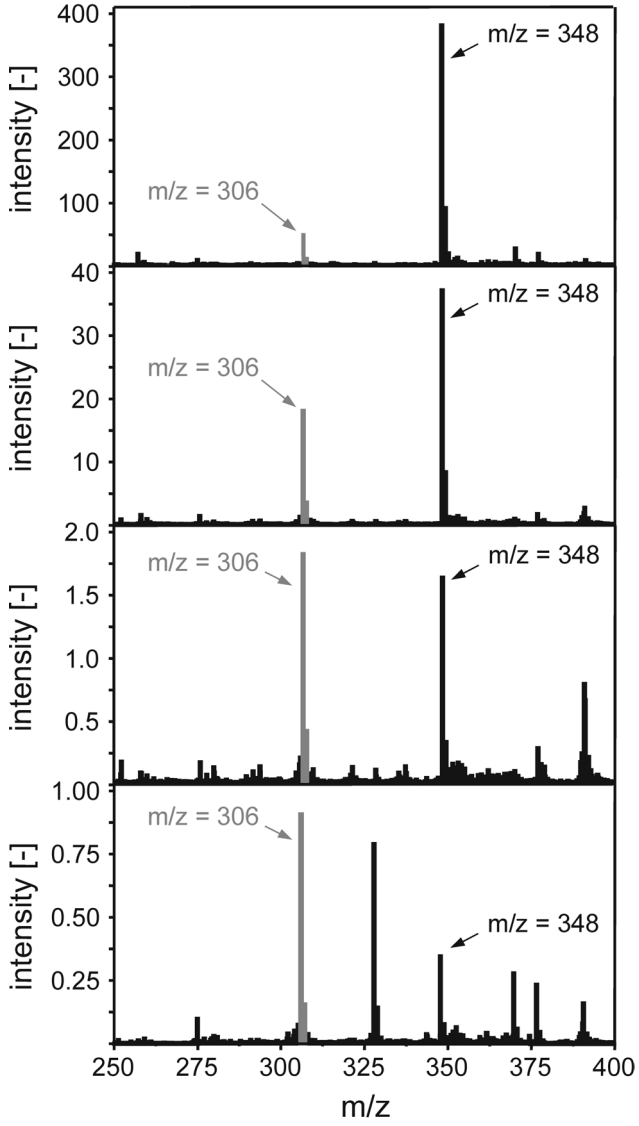

Figure 3.10: Mass spectra averaged over three minutes accumulation time measured at different total flow rates (from top to bottom: 2 μL/min, 0.5 μL/min, 0.2 μL/min and 0.1 μL/min). Indicated are the signals for the substrate and the product.

Due to this steady-state of the reaction, mass spectra can be acquired over minutes time span for each reaction time. When the total flow rate is decreased, the absolute signal intensity for product and substrate is also significantly decreased. However, the ratio of product to substrate signal intensity at each time can be taken as a measure of the reaction progress.

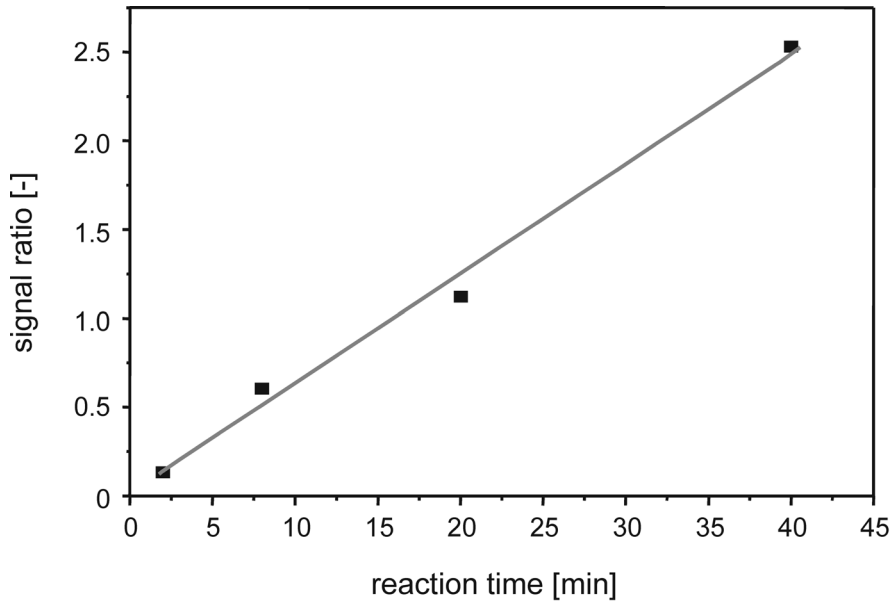

Figure 3.11: Ratio of product to substrate signal intensity versus different reaction times.

The plot of the signal ratio versus the reaction time provides a reaction profile and exhibits a straight line in the range between 2 minutes and 40 minutes reaction time, thus demonstrating the working range of the chip-reactor. Therefore, it can be concluded that the use of chip-based micro reactors for investigations on enzymatic reactions is well possible.

3.4 Conclusions

It can be concluded that ESI-MS is generally a useful method for the monitoring of enzyme-catalyzed reactions. The three different experimental set-ups investigated in the course of this work all proved to be useful for studying those conversions. The at-line approach provides full, time-resolved reaction profiles, which contain the highest degree of information, but are also rather time-consuming. The on-line approach proved to be feasible for the fast quantification of enzymatic activity. In a further advancement of the on-line approach, the use of a chip-based micro-reactor was investigated. This on-chip approach was demonstrated to provide information about the reaction progress even on the microscale.

In the comparison of the ESI-MS methodology with an independent optical method, the EALL assay technique, it becomes obvious that the EALL technique may already be carried out on a simple and cheap standard fluorescence spectrometer, although a time-resolved fluorescence spectrometer with microsecond resolution provides superior results. The ESI-MS method is advantageous in terms of the possibility of monitoring the substrate and product concentrations simultaneously. Therefore, two sets of data points are obtained in one single experiment. Furthermore, the ESI-MS method is, due to its high selectivity, likely to exhibit less interferences in complex matrices. Both detection methods are simple and comparably robust, and good results are obtained already under the optimum conditions for the enzymatic conversion. It can therefore be expected that both methods may find increased use in the future.

3.5 References

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Chapter 4

Simultaneous Determination of Enzymes by ESI-MS and Fluorescence Assays[‡]

A comparative investigation on the simultaneous determination of two enzymes by means of fluorescence and ESI-MS-detection is presented. As model system, the following reactions are used: The alkaline phosphatase (aP)-catalyzed reaction with 5-fluorosalicyl phosphate (5-FSAP) yields the fluorescent 5-fluorosalicylic acid (5-FSA), whereas microperoxidase 11 (MP-11) reacts with 4-(N-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) and H_2O_2 to the strongly fluorescent 4-(N-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA). As the emission spectra of the fluorescent products as well as the molecular masses of substrates and products do not interfere with each other, is it possible to monitor both reactions in parallel with both detection schemes. The fluorescence detector was coupled in-line with an ESI mass spectrometer so that both detection schemes can be directly compared. The measurements resulted in the same limits of detection, limits of quantification and linear ranges of the single/simultaneous enzyme determination for fluorescence and MS detection. While the relative standard deviations were significantly lower in case of fluorescence detection (1.4%-3.2%) than in mass spectrometry (5.7%-10.1%), the latter proved to be the more versatile approach for multianalyte determination.

[‡] C. Hempen, A. Liesener, U. Karst, accepted for publication in *Anal. Chim. Acta* **2005**.

4.1 Introduction

With the usually high demands on the bioanalytical methodologies regarding the number of samples that have to be analyzed, it is desirable to improve enzymatic assays with respect to throughput. Thus, the simultaneous identification and/or quantification of several analytes using simple and readily available instrumentation is desirable. In case of the most frequently applied conversion of colorless substrates to colored products and subsequent UV/vis detection, there are no possibilities for simultaneous multianalyte determination. Fluorescence-based detection schemes are generally better suited for the employment in multiplexed assays. Prerequisites for parallel fluorescence measurements are non-overlapping emission bands of the fluorescent products [1,2]. Recently, a multiplexed detection scheme for two different enzymes, a phosphatase and a peroxidase, based on microtitration plates fluorescence measurements of the fluorophores 5-fluorosalicylic acid (5-FSA) and 4-(N-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA) has been introduced [3]. However, the use of fluorescence detection in multiplexed assays is generally challenging, due to the high demands on the spectroscopic properties of the substrate and the respective product. Thus, the number of suitable compounds is very limited.

An alternative approach for monitoring two independent enzyme-catalyzed conversions is offered by employing electrospray ionization mass spectrometry (ESI-MS). Since in MS different compounds are separated solely by their mass-to-charge (m/z) ratio, this detection method is independent from the spectroscopic properties of the analytes. Therefore, a

multiplexed analysis without further separation is generally possible as long as the substrate and product species present in the assay exhibit different m/z values. The first approaches to a multiplexed analysis were presented by the group of Gelb and colleagues. They developed an assay scheme, which was based on the coupling of affinity chromatography for the extraction of the substrate and product from the reaction mixture with ESI-MS for the parallel quantification of those compounds. This assay scheme allowed the parallel assessment of enzymatic activities in cell lysates providing a tool for the diagnosis of enzyme deficiency related diseases [4–9]. Basile et al. developed a direct flow-injection ESI-MS assay, which allowed the *in vivo* differentiation of bacteria by their aminopeptidase activity profiles [10]. These profiles were generated by incubation of living bacteria cells with a mixture of different target-enzyme specific substrates and the quantification of substrate and product compounds after a defined incubation time. Four sorts of bacteria were used as model compounds, and each was found to exhibit different enzymatic activities towards the substrate compounds resulting in unique activity profiles.

In this chapter, a comparative study on the simultaneous quantitative determination of the two enzymes alkaline phosphatase and microperoxidase 11 is presented. The feasibility of the simultaneous MS-detection method was investigated using the fluorescence-based enzymatic assay as independent reference method. Therefore, the parallel quantification of the model enzymes was performed by means of direct flow-injection analysis (FIA) measurements on a coupled fluorescence and mass spectrometric detection system. The

reactions were carried out at-line generating time resolved reaction profiles. Besides the possibility for quantification of the enzyme content present, these profiles provided additional information about the working mechanism of the enzymes used in this study.

4.2 Experimental

Chemicals

All chemicals were purchased from Aldrich (Steinheim/Germany), Merck (Darmstadt/Germany) and Fluka (Neu-Ulm/Germany) in the highest quality available. Acetonitrile and water for the flow-injection measurements were LC-MS grade and purchased from Biosolve (Valkenswaard/The Netherlands). The microperoxidase MP-11 and alkaline phosphatase aP were purchased from Sigma (Deisenhofen/Germany). The syntheses of 4-(N-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) and 5-fluorosalicyl phosphate (5-FSAP) were performed as described in literature [11, 12].

Instrumentation

The flow infection measurements with fluorescence and MS-detection were performed with the following system: two LC-10AS pumps, degasser GT-154, SIL-10A autosampler, software Class LC-10 version 1.6, CBM-10A controller unit and RF-10AXL fluorescence detector (all components from Shimadzu, Duisburg/Germany). The injection volume was 5 μL for all experiments. As carrier stream, a mixture (v/v, 50/50) of methanol and 15 mM NH_4Ac buffer (pH 7.3) at a flow rate of 0.3 mL/min was used.

The fluorescence for 5-FSA was measured at excitation and emission wavelengths of 313 nm and 418 nm, respectively. For MNBDA, the excitation wavelength was set to 470 nm and the emission at 545 nm. The subsequent MS detection was performed by means of an esquire 3000^+ ion trap mass spectrometer controlled by EsquireControl V5.1 software from Bruker Daltonics (Bremen/Germany). All MS results were obtained using electrospray ionization (ESI) in the negative ion mode. Mass spectra were recorded in the full scan mode, scanning from m/z 50 to m/z 1000 employing the ESI-MS parameter settings as shown in table 4.1.

Table 4.1: List of ESI-MS parameters for Esquire 3000^+

Parameter	Settings
Nebulizer gas (N_2) pressure	45 psi
Dry gas (N_2) flow	10.0 L/min
dry gas (N_2) temperature	365 °C
Capillary high voltage	2049 V
capillary exit voltage	-102.6 V
skimmer voltage	-40.0 V
octopole 1 voltage	-12.0 V
octopole 2 voltage	-1.7 V
octopole amplitude	119.4 Vpp
Lens 1 voltage	5.0 V
Lens 2 voltage	60.0 V
trap drive level	33.6

Alkaline phosphatase (aP) assay

For the single determination of aP according to [13], 300 µL of a 5-FSAP solution ($1*10^{-3}$ mol/L; acetonitrile) were pipetted to 300 µL of an aP solution ($5*10^{-4}$ u/mL to 0.1 u/mL; NH_4Ac buffer; pH 7.3; 15 mM). Subsequently,

950 µL NH_4Ac buffer were added. Afterwards, every 2 min (20 times) 50 µL of the reaction solution were pipetted to 950 µL methanol in order to stop the enzymatic conversion, thus preserving the ratio of substrate to product as present at the respective time. Samples of every reaction time were injected in triplicate into the flow-injection set-up as described above.

Microperoxidase (MP-11) assay

For the single determination of MP-11 according to [3], 0.9 mL of a MNBDH solution ($5*10^{-4}$ mol/L) were added to 7.5 mL of a 30 mM solution of H_2O_2. 550 µL of this mixture were pipetted to 1000 µL of a solution of MP-11 ($1*10^{-9}$ mol/L to $2*10^{-7}$ mol/L) in NH_4Ac buffer (pH 7.3; 15 mM). Afterwards, every 2 min (20 times) 50 µL of the reaction solution were pipetted to 950 µL methanol in order to stop the enzymatic conversion. Samples of every reaction time point were injected in threefold into the flow-injection set-up as described above.

Simultaneous aP and MP-11 assays

The assays of the enzymes aP and MP-11 were performed simultaneously in a variation of the method published in [3]. Therefore, the respective reactions were executed similarly to the reaction described above. For each reaction solution, 150 µL aP ($1*10^{-3}$ u/mL to 0.2 u/mL; NH_4Ac buffer; pH 7.3; 15 mM), 150 µL FSAP ($2*10^{-3}$ mol/L; acetonitrile) and 475 µL buffer were mixed with 500 µL MP-11 ($2*10^{-9}$ mol/L to $4*10^{-7}$ mol/L) and 275 µL of the MNBDH/H_2O_2 mixture (0.9 mL of MNBDA; $1*10^{-3}$ mol/L / 7.5 mL H_2O_2; 60 mM). The concentrations of all enzyme and substrate solutions were twice as high as for

the single determinations of aP and MP-11, so that all final concentrations were equal. The aP solution with the highest concentration was added to the lowest MP-11 concentration. For all other reaction mixtures, the aP-concentration was decreased, whereas the MP-11 concentrations were increased. Again, every 2 min (20 times) 50 μL of the reaction solution were pipetted to 950 μL methanol in order to stop the enzymatic reactions. Samples of every reaction time were injected in triplicate into the flow-injection set-up described above. The fluorescence of the respective products was read out subsequently, due to technical limitations of the fluorescence detector. The MS detection of MNBDA and 5-FSA was performed truly simultaneously.

Investigations on early stage MP-11 reaction kinetics

In order to obtain time-resolved information about the early stages of the MP-11-catalyzed reaction fluorescence measurements in quartz cuvettes were performed using an Aminco Bowman AB2 luminescence spectrometer from Polytec (Waldbronn/Germany) with software version 5.00. For this reaction 2584 μL NH_4Ac buffer (pH 7.3; 15 mM), 200 μL MP-11 ($2*10^{-8}$ mol/L), 200 μL MNBDH ($5*10^{-4}$ mol/L) and 16 μL H_2O_2 were mixed in a cuvette. Approximately every 5 seconds, the fluorescence intensity of MNBDA fluorescence was measured with an excitation wavelength of 470 nm and an emission wavelength of 545 nm.

4.3 Results and discussion

4.3.1 Reaction properties

Goal of this work was to compare the results of fluorescence detection with ESI-MS-detection by expansion of the reaction scheme to a flow-injection system. Quantification of alkaline phosphatase (aP) was achieved by means of the reaction with 5-FSAP to the strongly fluorescent 5-FSA, whereas microperoxidase 11 (MP-11) could be determined by the conversion of the non-fluorescent MNBDH into the strongly fluorescent MNBDA as depicted in figure 4.1.

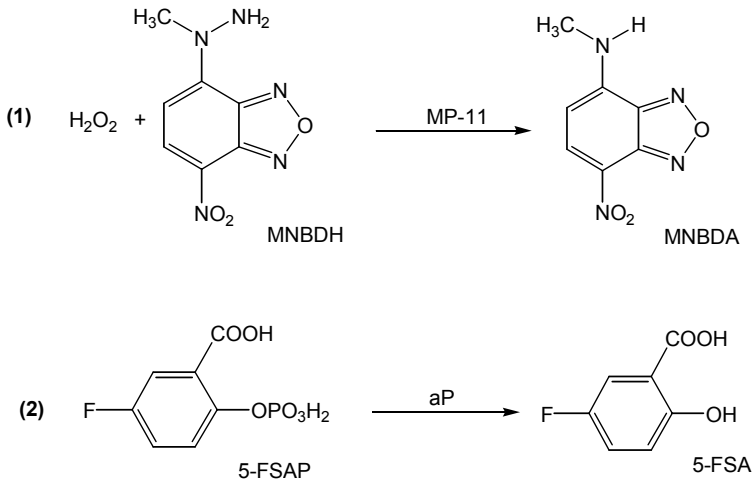

Figure 4.1: Enzymatic reactions for the determination of MP-11 (1) using MNBDH and for the determination of aP (2) using 5-FSAP.

The reactions were carried out both individually and simultaneously. While both individual reactions were already analyzed earlier using fluorescence spectroscopy, mass spectrometric detection has not been described for the analysis of either of these reactions. The reaction substrates were mixed manually and after approximately every 2 min, a sample aliquot (50 µL) of the reaction solution was pipetted into a vial with an excess of methanol in order

to stop the reaction and to obtain information about the progress of the two reactions over a period of 40 minutes (20 samples). This was performed for eight concentrations of each enzyme in the case of individual determinations as well as for simultaneous measurements with the second enzyme. Afterwards, the samples were injected into the FIA-system and analyzed by means of the fluorescence detector and subsequently by means of the coupled mass spectrometer.

A drawback of fluorescence detection in this case is that it is not possible to switch between different excitation and emission wavelengths during one single run. Consequently, two injections of the simultaneous reaction solution, with different detection wavelengths, had to be performed, thus not allowing a truely simultaneous detection of both reaction products. In contrast, the ESI-MS-detection enabled the simultaneous determination of aP and MP-11, since substrates and products of both reactions can be determined in parallel as shown by the following mass spectrum.

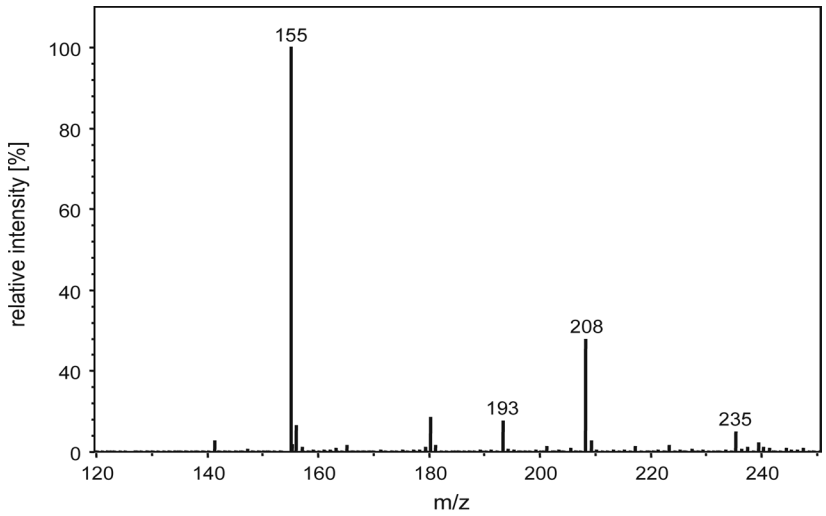

Figure 4.2: ESI-MS spectrum showing the signals of the educts (5-FSAP: 235; MNBDH: 208) and the products (5-FSA: 155; MNBDA: 193) of a simultaneous enzyme reaction (c_{aP} = 5 u/L; c_{MP-11} = 2*10^{-8} mol/L).

Figure 4.2 shows a mass spectrum of the simultaneous determination of the two enzymes, with an aP concentration of 5 u/L and an MP-11 concentration of $2*10^{-8}$ mol/L. The signals at m/z 155 and m/z 235 correspond to the deprotonated 5-FSA and 5-FSAP, respectively. Furthermore, the signals at m/z 193 and m/z 208 are assigned to the deprotonated MNBDA and MNBDH.

4.3.2 Figures of merit

A comparison of the figures of merit for the single and parallel determination of aP and MP-11 by means of fluorescence and ESI-MS detection after FIA is presented in table 4.2.

Table 4.2: Figures of merit for the single and simultaneous determination of the enzymes aP and MP-11 after a reaction time of 18 minutes by fluorescence and ESI-MS detection (n = 3)

Fluorescence Detection				
Assay	**LOD**	**LOQ**	**Linear range**	**RSD [%]**
aP_{Single}	$2*10^{-3}$ [u/mL]	$6*10^{-3}$ [u/mL]	$6*10^{-3}$-$5{\cdot}10^{-2}$ [u/mL]	3.2
$aP_{Simultaneous}$	$5*10^{-3}$ [u/mL]	$1.5*10^{-2}$ [u/mL]	$1.5*10^{-2}$-$5{\cdot}10^{-2}$ [u/mL]	1.7
$MP\text{-}11_{Single}$	$5*10^{-9}$ [mol/L]	$1.5*10^{-8}$ [mol/L]	$1.5*10^{-8}$-$2{\cdot}10^{-7}$ [mol/L]	1.9
$MP\text{-}11_{Simultaneous}$	$1*10^{-9}$ [mol/L]	$3*10^{-9}$ [mol/L]	$3*10^{-9}$-$1{\cdot}10^{-7}$ [mol/L]	1.4

ESI-MS Detection				
Assay	**LOD**	**LOQ**	**Linear range**	**RSD [%]**
aP_{Single}	$2*10^{-3}$ [u/mL]	$6*10^{-3}$ [u/mL]	$6*10^{-3}$-$5{\cdot}10^{-2}$ [u/mL]	6.3
$aP_{Simultaneous}$	$5*10^{-3}$ [u/mL]	$1.5*10^{-2}$ [u/mL]	$1.5*10^{-2}$-$5{\cdot}10^{-2}$ [u/mL]	5.7
$MP\text{-}11_{Single}$	$5*10^{-9}$ [mol/L]	$1.5*10^{-8}$ [mol/L]	$1.5*10^{-8}$-$2{\cdot}10^{-7}$ [mol/L]	8.3
$MP\text{-}11_{Simultaneous}$	$1*10^{-9}$ [mol/L]	$3*10^{-9}$ [mol/L]	$3*10^{-9}$-$2{\cdot}10^{-7}$ [mol/L]	10.1

The limits of detection, the limits of quantification and the linear ranges are determined for the reaction time of 18 minutes, whereas the RSD is calculated for all measurements of each enzyme at all concentrations and times (n = 960 for the single/parallel fluorescence measurements and for the single MS determination; n = 480 for the simultaneous MS measurement). The values for the LOD, the LOQ and the linear range of the MS-detection correspond to the results for the fluorescence detection. Both detection methods yield a LOD of $2*10^{-3}$ u/mL, a LOQ of $6*10^{-3}$ u/mL and a linear range from $6*10^{-3}$ u/mL to $5*10^{-2}$ u/mL for the single aP determination. For the simultaneous measurement with MP-11, the LOD and LOQ of aP are higher by approximately a factor of 2. In case of the single MP-11 determination, the LOD is $5*10^{-9}$ mol/L and the LOQ $1.5*10^{-8}$ mol/L. The linear range is observed from $1.5*10^{-8}$ mol/L to $2*10^{-7}$ mol/L. The figures of merit for the simultaneous measurements of MP-11 are lower by a factor of five for the fluorescence as well as for the MS detection. By comparing the relative standard deviations for all measurements, it can be observed that the values are much lower, especially in case of the simultaneous measurements for fluorescence detection. The RSDs for the single determination of aP and MP-11 are lower by factor 2 and 4, respectively, whereas the RSDs for the parallel measurements differ in factor 3 and 10 for aP and MP-11.

4.3.3 Comparison of the reaction progresses

In figures 4.3 and 4.4, the comparative injection profiles for the reaction of aP (c_{aP} = 10 u/L) with 5-FSAP and for the reaction of MP-11 ($c_{MP\text{-}11}$ = $2*10^{-7}$ mol/L) with H_2O_2 and MNBDH are presented. In all cases, the signals for the

different reaction times of the single determinations are shown. The upper profile displays the fluorescence detection traces of the products 5-FSA (313 nm/418 nm) and MNBDA (470 nm/545 nm), whereas in the lower profile the ESI(-)-MS signal traces for 5-FSA (m/z 155) and MNBDA (m/z 193) are shown. In figure 4.3, a typical kinetic behavior of an enzymatic reaction can be observed for the fluorescence as well as for the mass spectrometric detection of 5-FSA. In the beginning of the reaction, the signals have a relatively low intensity, which increases and reaches a maximum value with longer reaction times.

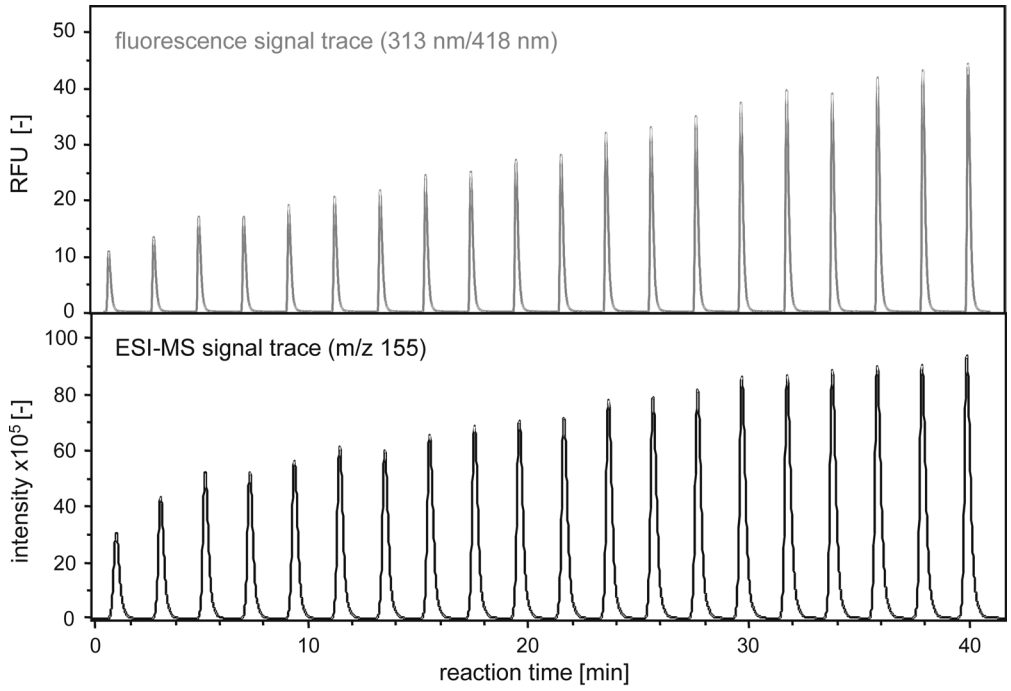

Figure 4.3: Comparative injection profile for the reaction of aP (c_{aP} = 10 u/L) with 5-FSAP over 40 minutes (reaction time between each signal approximately 2 minutes). In the upper trace, the fluorescence detection of the product 5-FSA (313 nm/418 nm) is shown, whereas the second trace shows the ESI(-)-MS detection for 5-FSA (m/z 155).

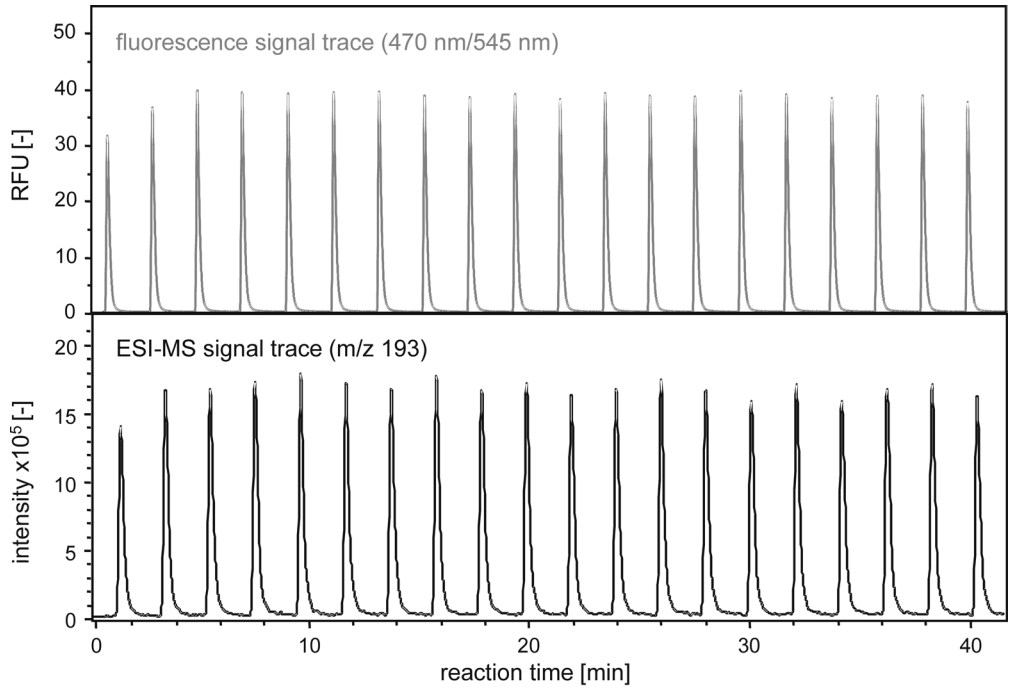

Figure 4.4: Comparative injection profile for the reaction of MP-11 (c_{MP-11} = 2*10^{-7} mol/L) with MNBDH and H_2O_2 over 40 minutes (reaction time between each signal approximately 2 minutes). In the upper trace, the fluorescence detection of the product MNBDA (470 nm/545 nm) is shown, whereas the second trace shows the ESI(-)-MS detection for MNBDA (m/z 193).

Figure 4.4 shows, in contrast, different kinetic properties for MP-11. The plateau, which is for aP reached after a reaction time of approximately 28 min, is in this case already achieved after 4 minutes, which means that the MP-11 reaction proceeds much faster than the aP reaction. The major difference in the kinetic behavior of aP and MP11 is the fact that the height of the plateau value in case of the MP-11-mediated conversion is strongly dependent on the concentration of MP-11, while the aP-catalyzed reactions are all running to the same plateau level. This indicates that MP-11 is inactivated after a certain number of conversions and thus not working like a true enzymatic catalyst as for instance aP.

4.3.4 Early-stage kinetics

To further investigate this kinetic behavior, the early stage of the reaction was monitored by means of fluorescence spectroscopy in a quartz cuvette, and a time-resolved reaction profile of the first 11 minutes was obtained.

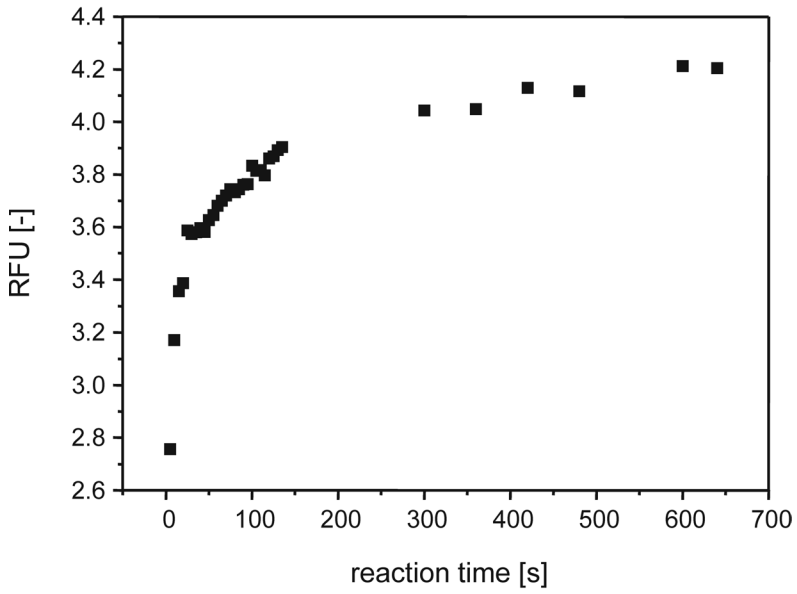

Figure 4.5: Kinetic behavior of the MP-11-mediated conversion of MNBDH and H_2O_2 in the early stage of the reaction ($c_{MP\text{-}11} = 2*10^{-8}$ mol/L).

It can be seen that the fluorescence intensity increases relatively steep in the first 2 minutes, whereas afterwards it rises very slowly so that a plateau is reached.

Spee et al. [12] have found for different microperoxidases to be relatively efficient catalysts for the reaction with aniline and H_2O_2. However, they also observed reaction profiles for the different conversions, which were characterized by a steep slope in the beginning and a rapid decrease of the reaction rate after 2 minutes, which is in accordance to the observations made here and which agrees with the results of the flow-injection measurements.

4.3.5 Comparison of single and simultaneous reaction properties

In order to further confirm that the monitoring of one enzymatic conversion by means of ESI-MS is not deteriorated by the second reaction proceeding simultaneously, the reaction profiles derived from a single enzyme assay and a simultaneous assay are compared.

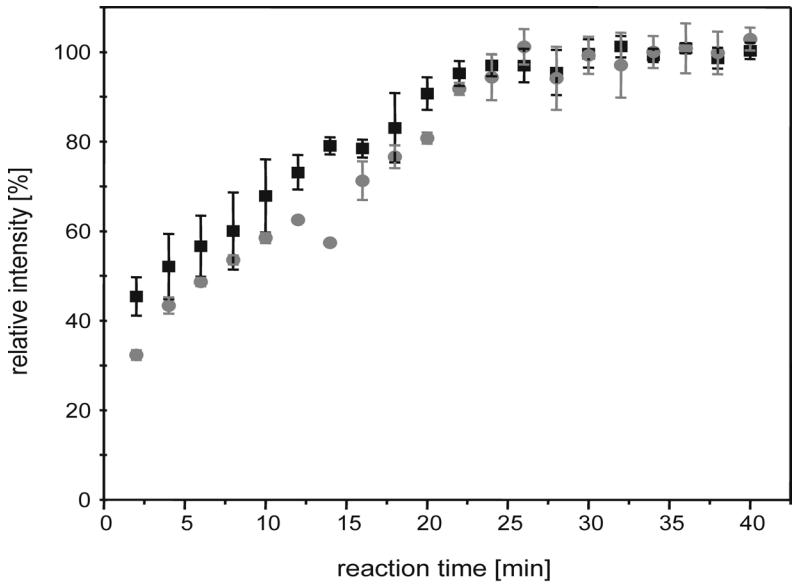

Figure 4.6: Comparison of the reaction profile of the single aP-reaction (■) with the profile of the simultaneous determination (●) (c_{aP} = 2*10^{-2} u/mL), both detected by means of ESI-MS.

It is observed that the development of the reaction product with time is rather similar for both reaction schemes. However, this observation approves the assumption that there is no interference of one enzymatic reaction with respect to the other.

4.4 Conclusions

A comparative study for the simultaneous determination of enzymes by means of flow-injection measurements with fluorescence and subsequently ESI-MS-detection is presented. The results of these investigations, which also provide mechanistical information on the reaction schemes, show that both detection methods do in principle allow the simultaneous determination of MP-11 and aP.

The advantage of fluorescence detection is a better reproducibility, which is expressed by lower RSDs. However, the reproducibility and accuracy of the ESI-MS detection method might be improved by employing an on-line sample clean-up feature (such as high turbulence liquid chromatography), in order to remove matrix components, which might have deteriorating effects on the ionization and thus the signal response.

Due to the technical properties of commercially available fluorescence detectors, which do not allow the detection at two different pairs of wavelengths simultaneously, it is advantageous to use MS-detection. This holds true especially with respect to the sample throughput demands in studies of enzymatic reactions, since analysis times can be reduced to half. Mass spectrometry additionally enables the detection of two reaction products as well as of the respective reaction educts at the same time providing a double set of data points, which can be used as control.

To sum up, it can be concluded that the parallel quantitative determination of enzymes by means of FIA/ESI-MS is well possible. A direct comparison with the fluorescence-based detection shows similarly performances of the assay schemes. Therefore, ESI-MS can be seen as a viable alternative to the fluorescence detection in enzymatic bioassays, with the advantage of being more versatile with respect to other enzymatic systems, since the physico-chemical demands on the properties of substrate and product compounds are only low.

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Chapter 5

Assessing Protease Activity Patterns by Means of Multiple Substrate ESI-MS Assays[‡]

The development of a simultaneous multiple substrate enzymatic assay based on electrospray ionization mass spectrometry (ESI-MS) detection is described. This multiplexed assay scheme was employed in a parallel proteolytic enzyme activity screening. As model systems, the respective activities of trypsin, thrombin, chymotrypsin, bromelain, ficin and elastase towards seven different substrates were assessed. The resulting activity patterns were evaluated semi-quantitatively, thus ranking the enzymatic activities in five classes of activity (very high, high, medium, low and no activity) with respect to the individual substrates. The validity of the MS-based multiplexed assay scheme was proven by comparison with the results obtained from single substrate assays detected by means of UV/vis absorption at 405 nm, showing good agreement of the resulting activity patterns and classifications.

[‡] A. Liesener, U. Karst, accepted for publication in *Analyst* **2005**.

5.1 Introduction

One of the major advantages of employing mass spectrometry (MS) as means of detection in enzymatic bioassays is the possibility to simultaneously monitor the conversion of several substrates in one reaction. There have been several different approaches to use this technique in enzymology and bioassays as described in chapter 2. However, up to now the predominant number of papers deals with single-substrate assays.

A first multiplexed assay system was presented by the group of Gelb and colleagues. A number of different target specific substrate conjugates were used to simultaneously probe for various enzymatic activities in cell lysates allowing the diagnosis of enzyme deficiency related diseases. The substrate conjugates were built up from an affinity tag, a linker and the enzyme target-specific substrate moiety. The affinity tag was used for the selective extraction of the substrates and products by means of affinity chromatography. Subsequently, the trapped analytes were eluted to the ESI-MS [1 – 7]. Pi et al. and Zea et al. presented multiplexed assay schemes for the competitive determination of enzyme/substrate specificity constants [8, 9]. Basile et al. developed an *in-vivo* multiplexed assay scheme for the differentiation of bacteria. A mix of four different substrates was incubated with living bacteria. Unique mass tags were released from each substrate during the reaction. The reaction mixture was filtered in order to remove the bacteria and transferred to the mass spectrometer. By determining the amount of each mass tag released in the mixture, a reactivity pattern for each stem of bacteria could be generated. These activity profiles were used as fingerprints in order to

differentiate the different types of bacteria [10]. Yu and co-workers employed a multiplexed assay scheme for the activity screening of different glycosidases. Small libraries containing nine carbohydrates of different masses were incubated with several glycosidases. The reaction mixture was analyzed by means of flow-injection analysis (FIA) ESI-MS. A general enzymatic activity towards a substrate was assessed upon monitoring decreasing substrate signal intensity and increasing signal intensity of the respective product [11]. A matrix-assisted laser desorption/ionization (MALDI) MS-based assay scheme for the activity profiling of kinases was presented by Min and colleagues [12]. A mixture of four different target specific substrates was immobilized on a MALDI target plate using a self-assembled monolayer. Several kinases were incubated separately on the target plate. After rinsing of the plate in order to remove the enzyme and addition of matrix, the MALDI-MS analysis was carried out. Enzymatic activity was assessed upon decrease of a substrate signal and build up of the according product signal.

The application of a multiplexed assay scheme for a fast, general enzymatic activity screening might be of special interest for investigations on the enzymatic behavior of complex biological mixtures such as snake venom, algae toxins or plant extracts. The multiplex assays scheme could therein be implemented as a fast pre-screening method in order to identify possible lead compounds. In such a multiplex screening, the activity of an enzyme towards several substrates is monitored. The generated “reactivity pattern” can directly be compared with the patterns generated under the same assay conditions with different enzymes to provide a functional characterization of the sample.

In the work presented in this chapter, the enzymatic activities of different proteases towards several substrates are investigated by means of direct flow-injection analysis (FIA) ESI-MS. Therefore, a multiplex assay employing seven different protease substrates was developed and the resulting activity patterns were evaluated. In order to validate the developed MS-based proteolytic assay scheme, control experiments based on UV/vis absorbance were performed and the results of both methods were compared.

5.2 Experimental

Chemicals

The substrates used in this work were obtained from Pentapharm Ltd. (Basel/Switzerland). Enzymes were obtained in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht/The Netherlands). All solvents were purchased in gradient grade quality from Biosolve (Valkenswaard/The Netherlands).

Flow-injection analysis (FIA)-MS instrumentation

To investigate the multi-substrate enzymatic reactions, a direct flow-injection system was set up. This FIA system comprising an autoinjector (SIL 10A), a controller unit (SCL 10Avp), an on-line degasser (DGU 14A) and two pumps (LC 10Advp) was coupled to an ESI single quadrupole MS (LCMS QP8000) controlled by LC Class 8000 V1.20 software (all from Shimadzu, Duisburg/Germany). All mass spectrometric measurements were carried out in the electrospray positive ion mode using the following conditions: Probe voltage 5 kV, curved desolvation line (CDL) voltage –35 V, deflector voltages

35 V, CDL temperature 230 °C, nebulizer gas (nitrogen) flow rate 4.5 L/min and a detector voltage of 1.6 kV. For detection of the signal traces of the substrates and their respective products, the selected ion monitoring (SIM) mode of the quadrupole MS was used. All substrates and reaction products were detected in the positive ion mode as their $[M + H]^+$ protonated molecular ions.

ESI-MS assay procedure

The experiments were carried out in aqueous solution without addition of buffer at ambient temperature. The enzyme solutions were freshly prepared by dissolving 1 mg of the respective protease in 25 mL of water. The substrate stock solutions were prepared individually in water to yield a substrate concentration of $1*10^{-3}$ mol/L. The substrate mix solution was prepared by combining 500 μL of each of the substrate stock solutions and filling up with water to 10 mL yielding a final concentration of $5*10^{-5}$ mol/L for every single substrate. For one experiment, 1 mL of the substrate mix solution and 1 mL of the respective enzyme solution were combined in a vial and shaken. The "reaction batch" was then placed in the autoinjector and samples of 5 μL were injected subsequently every 2.5 minutes into the carrier stream, which was set to 0.3 mL/min. The solvent composition of 80/20 (v/v) methanol/water was selected in order to ensure the stop of the reaction at the time of injection.

UV/vis-based assay procedure

All control experiments were performed as single-substrate assays on 96-well plates (Emergo, Landsweert/The Netherlands) using a Spectramax 250 platereader (Molecular Devices, Munich/Germany). In order to facilitate a comparison between the MS-based and absorption-based methods, similar reaction conditions were chosen. For each experiment, 100 µL of the respective enzyme solution and 100 µL of substrate solution (substrate stock solution diluted 1:20 with water yielding a final concentration of $5*10^{-5}$ mol/L) were combined. The progress of the reaction was followed by measurement of the PNA absorbance at 405 nm wavelengths over 65 minutes at room temperature.

5.3 Results and discussion

5.3.1 Properties of the multiplexed assays

As model proteases for the multiplex assays, trypsin, chymotrypsin, thrombin, bromelain, ficin and elastase were selected. The prerequisite for the choice of substrate compounds is that there is no overlap in m/z ratios of the different substrates and the respective products. The multiplex assay was developed following the reaction scheme presented in figure 5.1.

The enzymatic conversion of the respective substrates occurs as a C-terminal cleavage, thus releasing the chromogenic *para*-nitroaniline group (PNA) and resulting in a color change of the reaction mixture as well as in a change of the molecular mass of the substrate compounds of 120 Da. The substrates

employed in this study and the signal traces for the detection of the substrates and their products are listed in table 5.1.

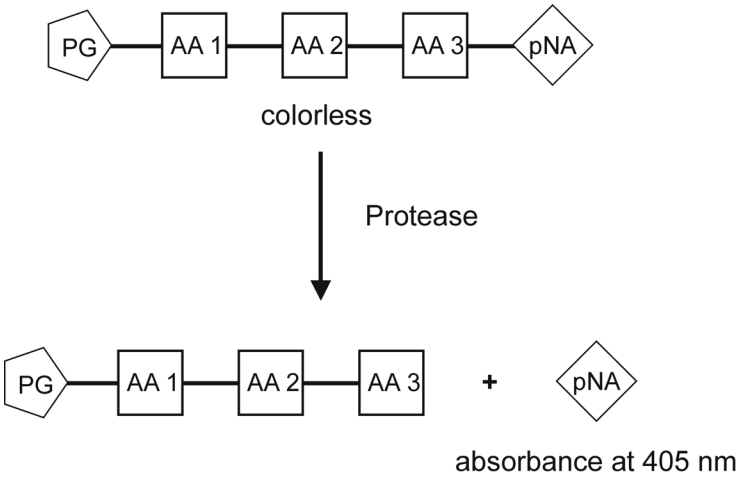

Figure 5.1: Reaction scheme of the multiplex proteolytic assays. The substrates are composed of a protecting group (PG), a varying sequence of amino acids (AA1 – AA3) to provide different selectivities and a chromogenic group (pNA), which is released by the enzymes. Due to different amino acid sequences, the substrates and the respective products are of different masses.

Table 5.1: List of substrates used in the multiplexed ESI-MS assays with the signal traces for the substrates and the products

Substrate Name	Substrate Structure	Substrate Signal Trace	Product Signal Trace
CHY	H-D-Ala-Pro-Phe-pNA	m/z 525	m/z 405
TRY	Bz-Val-Gly-Arg-pNA	m/z 555	m/z 435
TG	H-β-Ala-Gly-Arg-pNA	m/z 423	m/z 303
TH	H-D-CHG-Ala-Arg-pNA	m/z 505	m/z 385
C1E	CH_3CO-Lys(Cbo)-Gly-Arg-pNA	m/z 656	m/z 536
FXa	CH_3OCO-D-CHA-Gly-Arg-pNA	m/z 563	m/z 443
PAP	3-PhPr-Pro-Phe-Arg-pNA	m/z 671	m/z 551

Monitoring the increase of product concentrations and the decrease of substrate concentrations in the reaction solution in the SIM mode by repeated direct injection of the reaction solution yielded a full reaction time profile as presented for the reaction of trypsin with the substrate TRY.

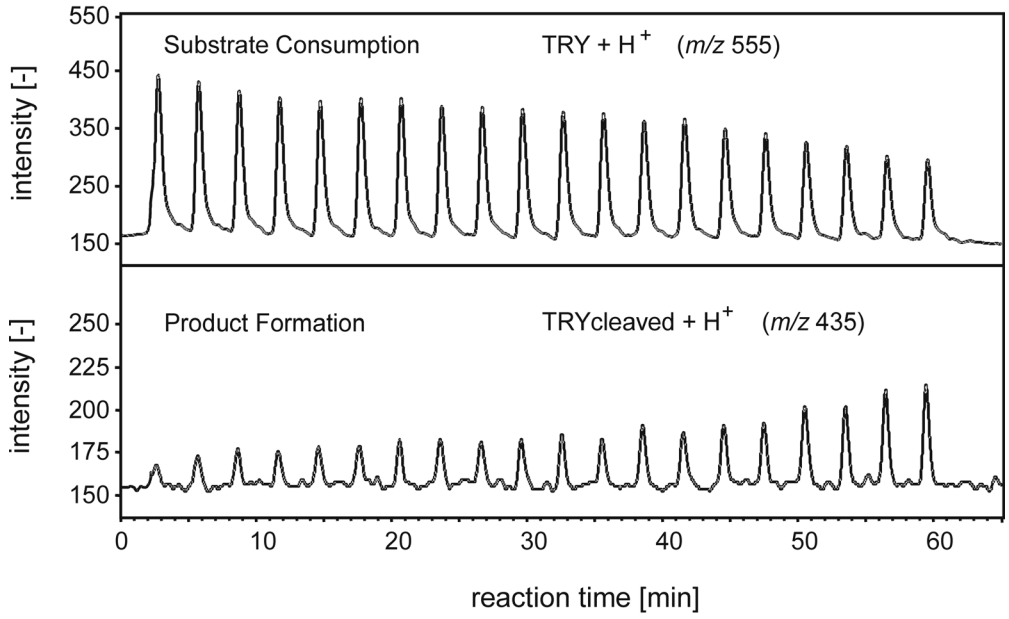

Figure 5.2: Injection profile of the trypsin-catalyzed hydrolysis of TRY; substrate (m/z 555) and product signal traces (m/z 435) are shown.

5.3.2 Generation of enzyme activity maps

All MS-based assays were carried out and evaluated in triplicate. The results of three selected multiplex assays (trypsin, thrombin and chymotrypsin) are depicted as reactivity maps in figure 5.3, which allow for a fast assessment of the respective enzymatic activities towards the different substrates.

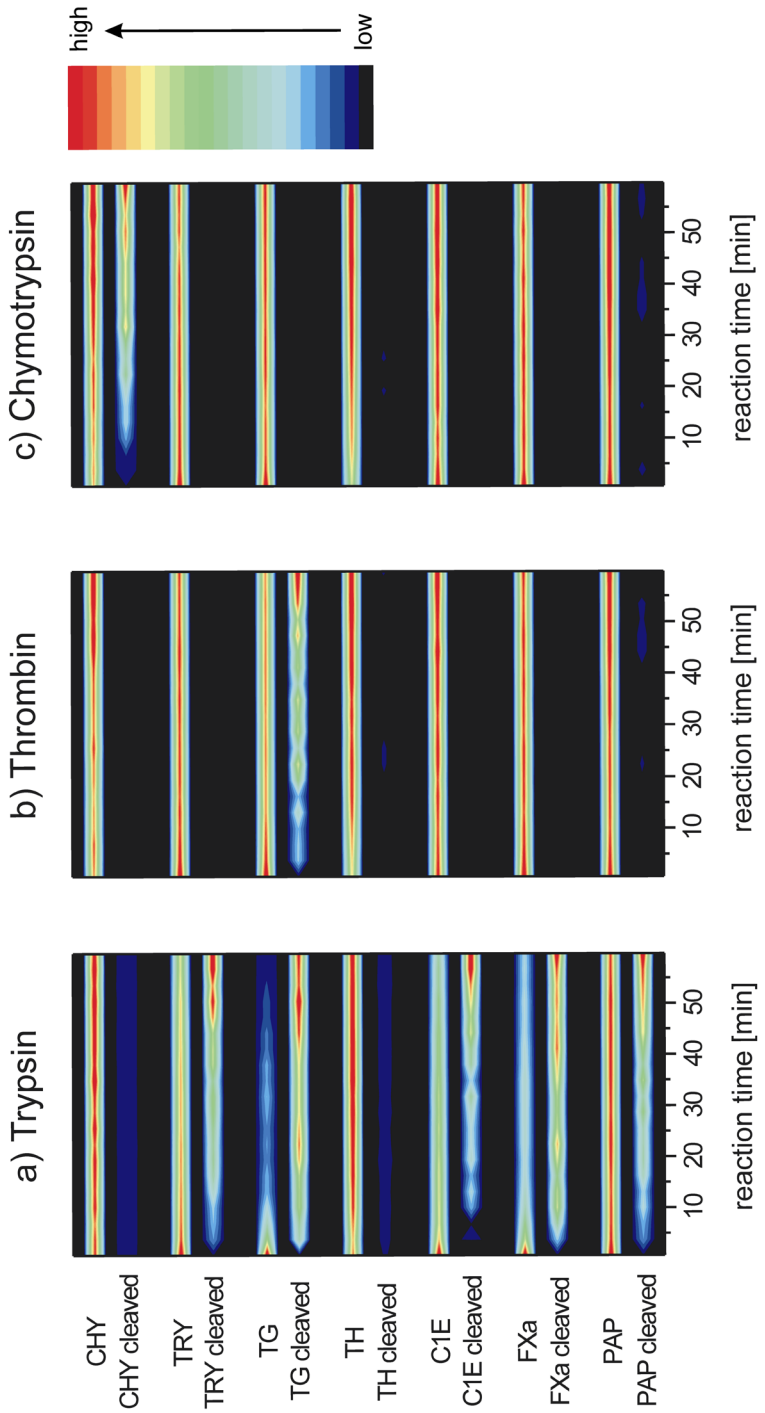

Figure 5.3: Activity maps for trypsin (a), thrombin (b) and chymotrypsin (c) derived from one multiplex assay each; plotted is the signal intensity (integrated peak area) related to the particular highest value for the respective substrate and product signal trace versus the reaction time (red → 100%; dark blue → 0.2%).

In order to condense the activity information of the multiplex assays into one simple chart, a two-dimensional contour plot was chosen with red color representing high and blue color representing low signal intensity. In the y-axis, the different m/z-traces observed are listed. The plot is extrapolated between the respective measurement points. The width of the signal traces promotes a better perceptibility of the slopes (rate of color change), which indicate the respective reactivity.

High enzymatic activity (fast reaction) is characterized by a steep slope in the substrate consumption and the product formation. For the trypsin assay, there is no decay of the CHY and TH signals and no increase of the respective product signals, indicating that there is no tryptic activity exhibited towards these substrates. For the other substrates, tryptic activity can be observed. The enzyme shows the highest activity towards TG and FXa, moderate activity towards TRY and C1E and comparatively low activity towards PAP. Similar reaction profile maps were obtained for the other five enzymes as well. Since the experiments were carried out under the same conditions, it is possible to directly compare the enzymatic activities of different proteases without the need of further calibration. Thus, it must be noted that the assertions about the enzymatic activity are only semi-quantitative (see table 5.2), allowing the comparison of different enzymes' activities, but not directly providing kinetic parameters.

5.3.3 UV/vis-absorption assays as reference method

In order to validate the MS-based multiplexed assays, single substrate assays using UV/vis-absorbance were performed on microplates for each protease. The assays were carried out eight-fold, and the mean values of absorbance of the reaction mixture at each time were plotted in a similar way as for the MS-based assays. Figure 5.4 shows the activity map of trypsin derived from six independent single substrate assay sets.

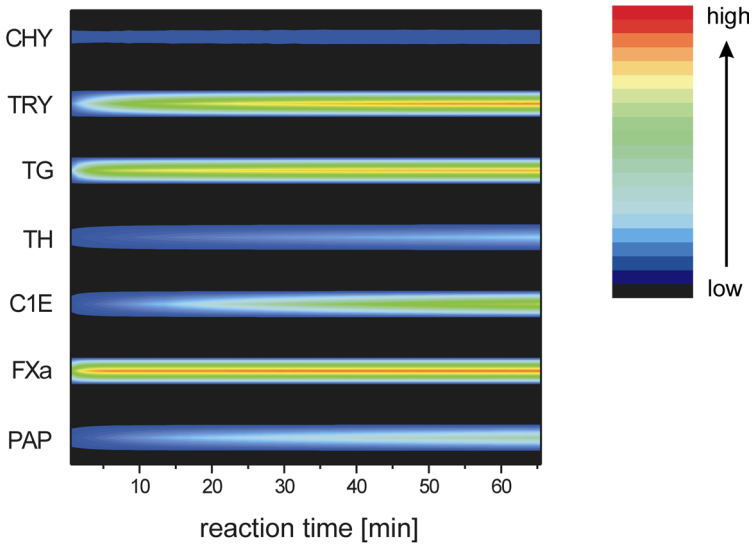

Figure 5.4: Reactivity pattern of trypsin derived from seven independent single substrate assay sets; plotted is the absorption measured at 405 nm related to the totally highest measured value (100% conversion) versus the reaction time (red → 100%; dark blue → 0.2%).

Evaluation of the UV/vis-based single substrate assays shows generally the same activity pattern for trypsin as described above for the MS-based multiplexed experiment. There is only one minor difference in the pattern, which is found for the conversion of C1E by trypsin, where a slightly higher activity is assessed through the MS-based assay. This deviation can probably

be contributed to the experimental error and the only semi-quantitative assignment of activity levels. The activity patterns derived for the other enzymes agree very well for both series of measurements (see table 5.2), indicating the validity of the MS-based assay scheme.

Enzyme	Enzymatic activity towards															
	CHY		TRY		TG		TH		C1E		FXa		PAP	
	MS	UV/vis	MS	UV/vis	MS	UV/vis	MS	UV/vis	MS	UV/vis	MS	UV/vis	MS	UV/vis
Trypsin	-	-	+++	+++	++++	++++	-	-	+++	++	++++	++++	+	+
Thrombin	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Chymotrypsin	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Bromelain	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ficin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Elastase	-	-	+	+	-	-	-	-	-	-	-	-	-	-

Table 5.2: Qualitative comparison of enzymatic activities towards the model substrates determined by MS-based multiplexing assays and UV/vis absorption-based single substrate assays (++++ : very high activity; +++ : high activity; ++ : medium activity; + : low activity; - : no activity)

5.3.4 Assessment of ion suppression effects

Additionally, the comparison of total signal intensities in the different MS-based assays provides information about the ion suppression caused by the different enzymes. Several studies showed that the presence of large, involatile biomolecules such as proteins is prone to interfere dramatically with the analyte ionization, thus resulting in a loss of signal intensity [4]. The comparison of the signal intensities of an unreacted substrate in the presence of different enzymes allows the qualitative assessment of the respective ionization suppression.

Table 5.3: Total signal intensities (in 10^3) of substrates TH, CHY and FXa in the presence of the different enzymes.

	TH	**CHY**	**FXa**
Trypsin	417 (± 11% RSD)	19 (±6% RSD)	n/a *
Thrombin	371 (± 6% RSD)	18 (±10% RSD)	168 (± 4% RSD)
Chymotrypsin	345 (± 4% RSD)	n/a *	165 (± 4% RSD)
Bromelain	494 (± 4% RSD)	34 (± 11% RSD)	225 (± 3% RSD)
Ficin	455 (± 6 RSD)	23 (± 12% RSD)	198 (± 3% RSD)
Elastase	481 (± 7% RSD)	32 (± 14% RSD)	222 (± 4% RSD)

*: not to be taken into account, since substrate consumption is taking place

When comparing the total signal intensities for the same amount of analyte in the presence of the different enzymes, the ion suppression potential of the individual protein is the higher, the lower the signal response of the analyte is. Therefore, when comparing the signal intensities of TH in the presence of the different enzymes, the relative order of the ion suppression potential can be concluded to be bromelain ≤ elastase < ficin < trypsin < thrombin < chymotrypsin. To validate this conclusion, the total signal intensities for CHY

and FXa in the presence of the enzymes are also compared. In case of CHY, the signal response in presence of chymotrypsin cannot be taken into account, since the substrate is converted. The same holds for the response of the FXa signal in presence of trypsin. Excluding the respective enzymes, the relative order of ion suppression potential of the enzymes is the same in all cases.

Sojo et al. describe another possible ion suppression effect, which can be caused by the co-elution of the substrate and product compounds and lead to erroneous results [5]. However, the generation of false positive information by a decrease in signal intensity for a substrate compound caused by suppression effects can be avoided by assessing the parallel development of the according product signal as performed here. An enzymatic conversion is not likely to occur without an increase of the respective product signal. The conclusion that ion suppression effects are not disturbing the ESI-MS assay performance is consolidated by comparison of the obtained results with the data derived from the UV-absorbance-based control experiments. The activity classification from both assays agrees in 41 out of 42 cases.

5.4 Conclusions

It could be demonstrated that the parallel monitoring of seven different substrate conversions in one single multiplexed assay is well possible for a broad range of different proteases. The validity of the approach was assessed by comparison with an UV/vis-absorption-based reference assay scheme. The classification of the enzymatic activities towards the different substrates results for both, the mass spectrometric multiplex as well as the optical single-substrate assays, in largely the same activity pattern. Ion suppression effects by the presence of the enzyme molecules during the analysis can also be identified, but do not seem to significantly affect the performance of the assay scheme.

The reactivity maps for each enzyme can be seen as a “fingerprint” and can be used for identification of enzymes by their reactivity pattern. In case of a screening for a compound with a certain enzymatic activity (e.g. thrombin-like) in a complex mixture of several enzymatic components, the reactivity pattern for the desired enzyme is first derived from one assay. After separation, the purified sample fractions can be subjected to the same assay, and the reactivity maps for each fraction can be compared with the target pattern allowing to identify the active compounds. This strategy is further discussed in chapter 6 in the application of the ESI-MS-based multiplex assay in the screening for proteolytic activities in snake venom.

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Chapter 6

Screening for Proteolytic Activities in Snake Venom by Means of a Multiplexed ESI-MS Assay Scheme

A multiplexed mass spectrometry-based assay scheme for the simultaneous determination of five different substrate/product pairs was developed as a tool for screening of proteolytic activities in snake venom fractions from Bothrops moojeni. The assay scheme was employed in the functional characterization of eight model proteases. Time-resolved reaction profiles were generated and the relative reaction progress at each time was determined. These were used to semi-quantitatively sort the catalytic activities of each enzyme towards the respective substrates into six classes. The resulting activity pattern served as an activity fingerprint for each enzyme.

The multiplexed assay scheme was then applied to a screening for proteolytic activities in fractions of the pre-separated venom from Bothrops moojeni. Activity patterns of each fraction were generated and used to classify the fractions into three different categories of activity. By comparison of the fingerprint activity patterns of the venom fractions and the model enzymes, a compound with proteolytic properties similar to activated protein C was detected.

6.1 Introduction

Enzymatic catalysis plays a key role in the regulation of all kinds of processes in life. Serine proteases like thrombin play a major role in the regulation of the blood coagulation system and are therefore an important target for the development of anti-thrombosis drugs [1]. Besides thrombin, a large number of other proteases and factors are involved in the regulation of the blood coagulation cascade, which can also be a potential target for pharmaceutically active compounds. Snake venom is accounted to be a major natural source for these substances, typically containing at least several hundred of different biologically active components [2 – 4]. In order to explore the different enzymatic activities found in these highly complex mixtures, a vast number of samples has to be analyzed. Therefore, the demand for a rapid and rugged activity screening method is high.

Typically, the determination of enzymatic activities is performed in single-substrate-single-enzyme assay schemes by either UV/vis-absorbance-, fluorescence- or radioactivity-based detection methods. Since the demands of an enzymatic activity screening with respect to sample throughput are high, a multiplexed assay approach is desirable in order to shorten analysis times. In contrast to the standard detection schemes used in enzymatic bioassays, mass spectrometry (MS) as a means of detection is prone to be used in a multiplexed assay approach as discussed in chapter 5.

Therefore, an ESI-MS-based multiplexed assay scheme seems to be a highly useful tool for the profiling of proteolytic activities in snake venom. Consequently, a multiplexed direct flow-injection analysis (FIA) ESI-MS assay was developed employing five different substrates as activity probes. The assay scheme was used to generate activity fingerprints for eight different model proteases. Subsequently, it was applied to screen for and to characterize the proteolytic activities in fractions of the pre-separated venom from *Bothrops moojeni*.

Figure 6.1: Picture of *Bothrops moojeni* in threat position [8]; milking of snake venom [9].

6.2 Experimental

Chemicals

The substrates, enzymes and snake venom fractions used in this study were kindly provided by Pentapharm (Basel/Switzerland). Due to patent right reasons, the model enzymes and substrates used in this study cannot be named explicitly. The model proteases A, B, C, D, E, F, G and H were obtained in the highest purity available. The p.a. grade ammonium acetate was obtained from Merck (Darmstadt/Germany). All solvents were purchased in LC-MS grade quality from Biosolve (Valkenswaard/The Netherlands).

Sample preparation

All enzyme and substrate solutions for the ESI-MS assays were prepared and stored at –18 °C prior to use. The five substrates (see table 1) were dissolved in buffer (15 mM ammonium acetate/ammonia, pH 7.5) yielding a mixed substrate solution with concentrations of $5*10^{-5}$ mol/L of each compound.

Table 6.1: List of substrates used in the multiplexed activity assays and the respective signal traces for the detection of substrates and the products.

Substrate	Substrate Signal Trace	Product	Product Signal Trace
V	m/z 505	Vcleaved	m/z 385
IV	m/z 541	IVcleaved	m/z 421
III	m/z 563	IIIcleaved	m/z 443
II	m/z 611	IIcleaved	m/z 491
I	m/z 654	Icleaved	m/z 534

All model enzymes were dissolved in buffer to yield solutions with a content of 100 μg/mL.

The venom of *Bothrops moojeni* was fractionated by preparative gel chromatography yielding fractions with venom components separated by their molecular masses. Fractionation of the venom was performed by Anna-Maria Perchuc from Pentapharm Ltd. (Basel/Switzerland). The gel fractions (F28 – F46) were frozen and kept at –18 °C prior to use.

Instrumental set-up of the FIA/ESI-MS system

For the FIA experiments, a flow-injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/Germany) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL/min of a 75/25 (v/v) mixture of methanol and water.

Mass spectrometric detection

For detection, an Esquire 3000^+ ion trap mass spectrometer (Bruker Daltonik, Bremen/Germany) equipped with an ESI source was used. All measurements were performed using the positive ion MS mode. Mass spectra were recorded over a range from m/z 100 to m/z 1000 in full scan mode. Instrumental settings are shown in table 6.2. The resulting data was analyzed using DataAnalysis software version 3.1 (Bruker Daltonik, Bremen/Germany).

Table 6.2: List of ESI-MS parameter settings

Parameter	Setting
nebulizer gas (N_2) pressure	40.0 psi
dry gas (N_2) flow	10.0 L/min
dry gas (N_2) temperature	365 °C
capillary high voltage	5000 V
capillary exit voltage	156.6 V
skimmer voltage	15.0 V
octopole 1 voltage	11.69 V
octopole 2 voltage	3.26 V
octopole amplitude	127.9 Vpp
lens 1 voltage	-6.1 V
lens 2 voltage	-49.8 V
trap drive level	57.0

ESI-MS assay procedure

All ESI-MS-based assays were performed according to the following general procedure: 1400 μL of the substrate mixture solution was pipetted into a 1.5 mL vial. The reaction was initiated by addition of 100 μL of the respective enzyme solution or gel fraction. Every two minutes over a period of 40 minutes, a 50 μL aliquot was sampled from the reaction mixture and quenched in 950 μL of methanol, thus stopping the reaction by rapidly denaturing of the enzyme. The quenched samples were subsequently injected in triplicate into the FIA/ESI-MS system and analyzed. The injection volume was set to 5 μL.

6.3 Results and discussion

6.3.1 Properties of the multiplexed screening

An ESI-MS-based multiplexed assay scheme for the screening and characterization of proteolytic activities in snake venom was developed. In the first step, the eight model proteases were assayed individually with a mixture of five different substrates. In order to monitor the simultaneous conversion of the substrates to the respective products, sample aliquots out of the reaction mixture were taken every two minutes over a time span of 40 minutes. The conversion in the sample aliquots was stopped by quenching with a 19-fold excess of methanol, thus conserving the state of product formation and substrate consumption at each time. The samples were analyzed by means of direct FIA/ESI-MS.

Figure 6.2 shows the mass spectra obtained for different reaction times in the assay with the model protease thrombin. In the beginning of the reaction (two minutes reaction time), there are only signals found, which correspond to the five substrates (see table 1). At the end of the reaction (40 minutes reaction time), the signals for the substrates V and I are no longer present, while the signals of the respective products Icleaved and Vcleaved can be found, indicating the complete conversion of these two substrates. The signal heigths for the substrates II, III and IV are also decreased over the course of the reaction and the respective product signals can be found, but the conversion of the substrates is not yet completed.

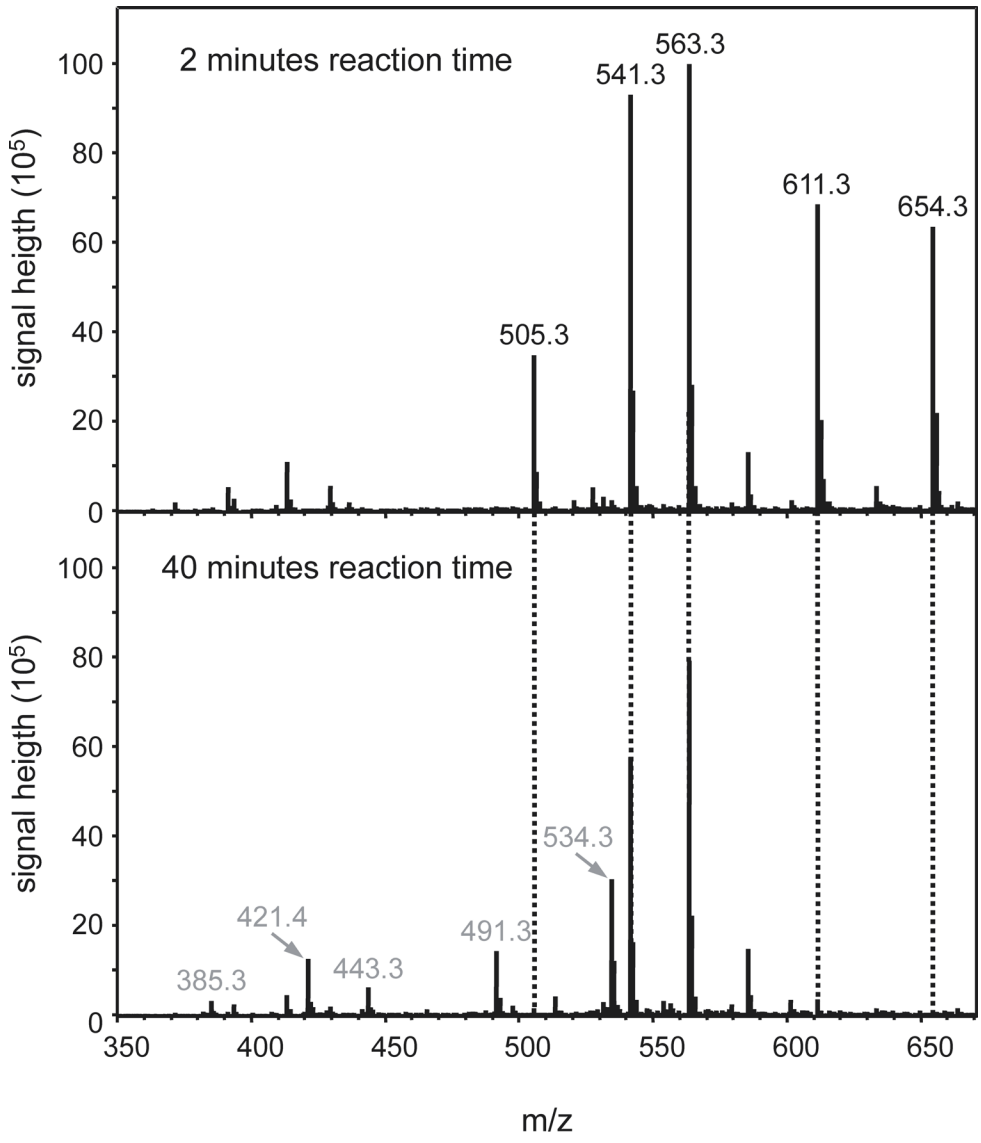

Figure 6.2: Mass spectra obtained from samples of the thrombin reaction mixture after 2 minutes and after 40 minutes reaction time. The mass spectrum after 2 minutes reaction time shows only signals for the substrates I (m/z 654.3), II (m/z 611.3), III (m/z 563.3), IV (m/z 541.3) and V (m/z 505.3). After 40 minutes reaction time signals for the respective products Icleaved (m/z 534.3), IIcleaved (m/z 491.3), IIIcleaved (m/z 443.3), IVcleaved (m/z 421.4) and Vcleaved (m/z 385.3) are observed, while the substrate signals intensity is decreased (position of substrate signals indicated by the dotted lines).

In order to generate time-resolved reaction profiles, the signal traces of the substrates and products are extracted and the area underlying the peaks is integrated resulting in values for the respective signal intensities. Plotting the peak areas for each substrate and the according product versus the reaction time yields the time-resolved reaction profiles of the respective conversions.

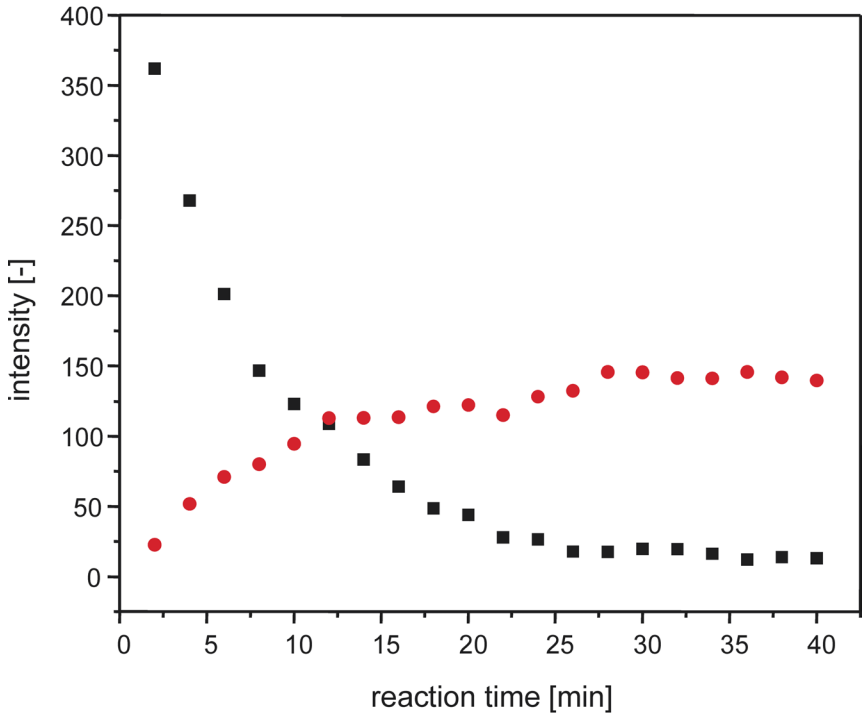

Figure 6.3: Time-resolved reaction profile of the conversion of substrate V catalyzed by model protease E. Substrate consumption (■) and product formation (●) curves are shown.

Figure 6.3 shows the complete reaction profile of the model protease E-catalyzed conversion of substrate V. The conversion is completed after 30 minutes reaction time, since the curves for both substrate consumption and product formation are reaching a stable plateau value.

6.3.2 Activity classification of model proteases

Quantification of analytes in MS is often achieved by addition of an internal standard. The concept of using the substrate as internal standard for the relative quantification of the product was discussed by several authors with respect to the use in MALDI-MS assay schemes *[5 – 7].* Based on their approach, the relative reaction progress (RRP) at a time was assessed by the ratio between product and substrate in the sample. The RRP in percent is expressed as the product signal intensity (S_P) divided by the sum of product plus remaining substrate signal intensity (S_S).

Equation 6.1: $$RRP(\%) = \frac{S_P}{S_S + S_P} * 100$$

The value for the RRP ranges from 0% (no product formed) to 100% (complete conversion of the substrate). Plotting the RRPs for the five parallel substrate conversions catalyzed by one enzyme versus the reaction time results in an “activity map” for the respective enzyme.

Figure 6.4 shows the activity map of the model protease E as a “heat map”, where red color represents high values (complete conversion) and blue color represents low values (no conversion; noise). In the y-axis, the different substrate conversions observed are listed. The plot is extrapolated between the respective measurement points. The width of the signal traces promotes a better perceptibility of the slopes (rate of color change), which indicate the respective reactivity. High enzymatic activity (fast reaction) is characterized by

a steep increase of the RRP value. Similar reactivity maps were obtained for the other enzymes as well.

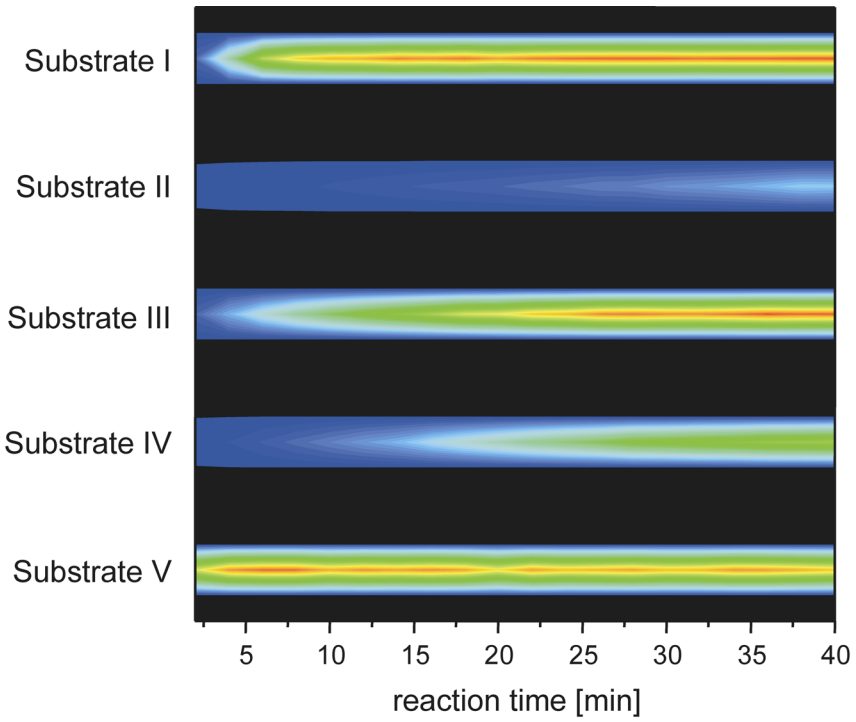

Figure 6.4: Reactivity map of the model protease E showing the relative reaction progress at each point in time for the conversion of the respective substrates (red → 100% RPP; dark blue → 0.2% RPP).

On the basis of these maps, the individual catalytic activity of the enzymes towards the respective substrates could be assessed. Therefore, the RRP values for each conversion at 20 minutes and 40 minutes reaction time were taken into account. As summarized in table 6.3, the data result in six classes of enzymatic activity.

Table 6.3: Criteria for classification of enzymatic activities by their time-dependent RPP values.

Activity Classes	RRP value after 20 min	RRP value after 40 min
very high activity	~80%	~100%
high activity	~40%	~100%
medium activity	~10%	~50%
low activity	~3%	~20%
very low activity	~1%	~3%
no activity	≤1%	≤1%

The complete overview of the activities of the eight model proteases is presented in table 6.4. This classification of the respective activities characterizes the enzymes and can be used as activity fingerprint.

Table 6.4: Summary of catalytic activity classes of each enzyme with respect to the different substrates (++++: very high activity; +++: high activity; ++: medium activity; +: low activity; +/-: very low activity; -: no activity).

Model Enzyme	I	II	III	IV	V
Protease A	-	+/-	-	-	-
Protease B	+/-	+/-	++	-	+/-
Protease C	++++	++++	++++	++++	-
Protease D	++++	++++	++++	+++	-
Protease E	++	++++	+++	+	-
Protease F	++++	+	+++	++	++++
Protease G	++++	++++	+	++++	++++
Protease H	++++	+++	+	+	+++

6.3.3 Screening for proteolytic activities in the venom fractions

In the second part of this work, fractions of the venom from *Bothrops moojeni* were screened for their proteolytic properties. Each fraction was assayed under the same conditions as the model enzymes, which allows a direct comparison of activities between the individual fractions and the model enzymes. The complete activity maps of all fractions are presented in figure 6.5.

In the y-dimension, the different gel fractions of the snake venom are listed from F46 to F28. The RRP time profiles for the conversions of each substrate are presented in the according columns. Thus, each line through the five columns represents the activity map for the individual venom fraction. According to table 3, the catalytic activity of each venom fraction towards the five substrates is assessed and classified. The results of this procedure are given in table 6.5.

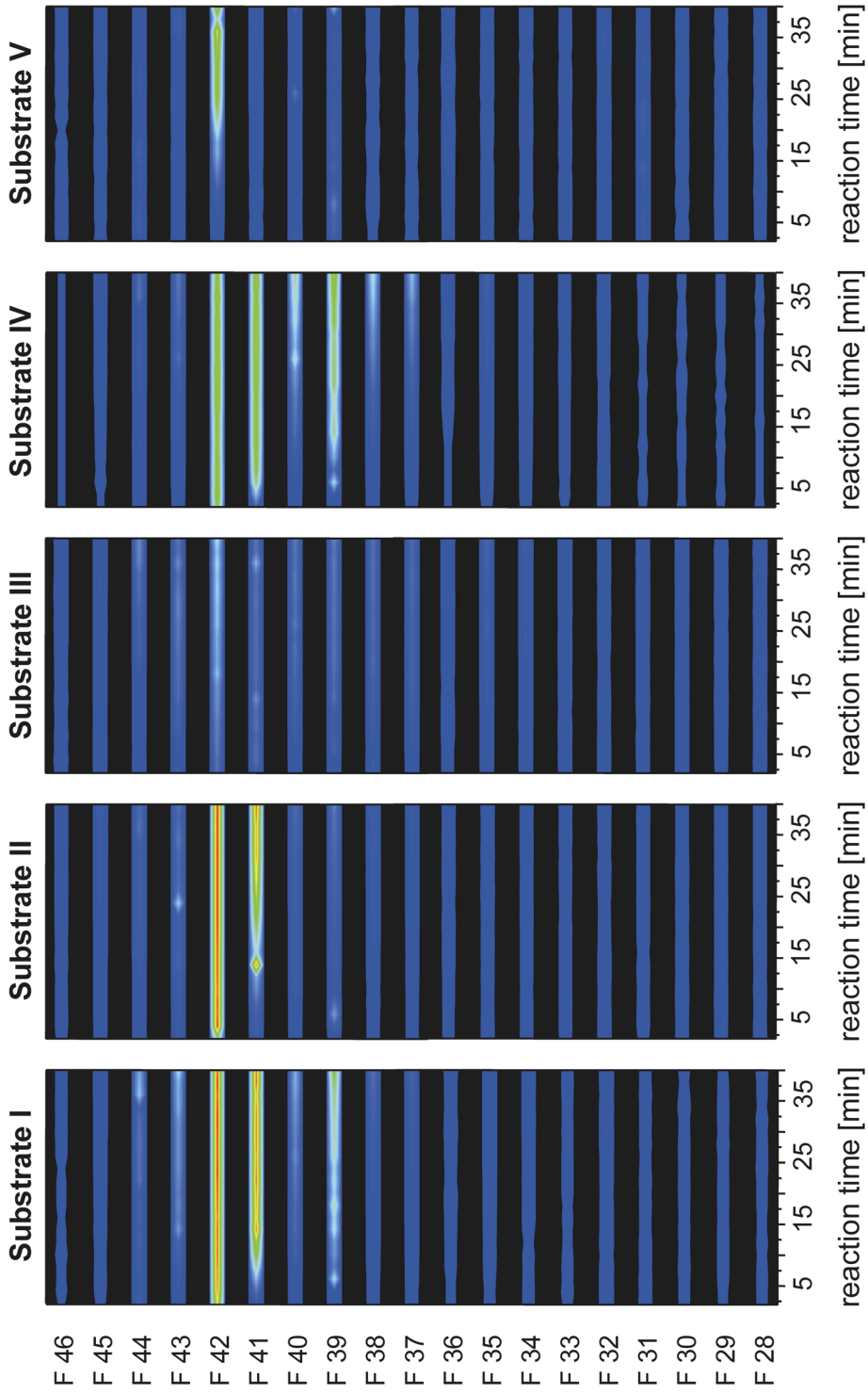

Figure 6.5: Complete activity maps for all venom fractions (F28 to F46) showing the time-resolved relative reaction progress of the respective substrate conversions mediated by the individual venom fraction (red → 100% RPP; dark blue → 0.2% RPP.

Table 6.5: Summary of catalytic activity classes of each venom fraction with respect to the different substrates (++++: very high activity; +++: high activity; ++: medium activity; +: low activity; +/-: very low activity; -: no activity).

Venom Fraction	I	II	III	IV	V
F 46	-	-	-	-	-
F 45	-	-	-	-	-
F 44	+	+/-	+	+/-	-
F 43	++	+	+/-	+	+/-
F 42	++++	++++	+	++++	++++
F 41	++++	+++	+/-	++++	+/-
F 40	+	+/-	+/-	++	-
F 39	++	+	+/-	+++	-
F 38	+/-	+/-	+/-	++	-
F 37	+/-	-	+	+	-
F 36	-	-	-	-	-
F 35	-	-	+/-	+/-	-
F 34	-	-	+/-	-	-
F 33	-	-	-	-	-
F 32	-	-	-	-	-
F 31	-	-	-	-	-
F 30	-	-	-	-	-
F 29	-	-	-	-	-
F 28	-	-	-	-	-

Based on this classification scheme, the venom fractions can be sorted into three different categories. Generally, proteolytic activity seems only to occur in the fractions F37 to F44 and F34 to F35. Consequently, the first category comprises the fractions with no proteolytic activity (F28 – F36, F45, F46). The second category, fraction F42, is characterized by significant catalytic activity towards the substrates I, II, IV and, most importantly, V, while the activity towards substrate III is only low. In fact, F42 is the only fraction, which efficiently catalyzed the hydrolysis of substrate V. The fractions F43, F44 and

especially F41 show some similarities to the activity profile of F42 and are therefore also selected to the same category. The third category is characterized by the activity pattern of F39. This fraction exhibits some catalytic activity towards the substrates I and IV and low activity towards substrates II and III, but most importantly, no activity towards substrate V. Somehow related patterns can be observed for the fractions F37, F38 and F40, which are therefore also aligned to category three.

The fact that several neighboring fractions seem to have a similar activity pattern with respect to the main fraction of a category can be explained by the separation. Gel permeation chromatography separates compounds by their molecular masses. However, the separation efficiency is not high and therefore, compounds of similar molecular masses are not sufficiently separated. This results in some active compounds being distributed in different amounts over several fractions. This conclusion is supported by the fact that the characteristic enzymatic activities all are decreasing around the dominant fraction.

The characteristic enzymatic activity fingerprints generated in these screening assays for the venom fractions can be compared with the activity profiles of the model proteases. As a result, one observes a high correlation in the patterns of F42 and model protease F. Therefore, the conclusion can be drawn that a protease F-like compound is present in a significant amount in the venom fraction F42.

6.4 Conclusions

The opportunity for multiplexed analysis of enzymatic activities by means of ESI-MS-based assay schemes offers great possibilities for the functional characterization of enzymes. The successful application of such a multiplex assay was demonstrated on the characterization of eight different proteases. The main advantage of this methodology lies in the application as screening tool for enzymatic activities in complex biological samples. By using this assay scheme, it was possible to rapidly identify and classify proteolytic activities in the venom fractions of *Bothrops moojeni*. The classification was carried out semi-quantitatively by determination of the relative reaction progress at defined reaction times. The resulting activity patterns could be used as fingerprints to characterize the respective venom fractions. Additionally, it was possible to find model-like activities in the venom fractions by comparison of the fingerprints.

This study clearly indicates the high potential of employing multiplex ESI-MS assays in enzymatic activity screening processes. However, it will be advantageous for the characterization of the enzymatic activities, and thus the unique character of the fingerprint activity patterns to widen the scope of substrates used in an assay. Further work will be directed to employ a broader range of different substrates to derive more detailed information from the multiplex screening.

6.5 References

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picture copyright by Cristiano Nogueira

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Chapter 7

ESI-MS-Based Screening for Acetylcholinesterase Inhibitors in Snake Venom

An electrospray ionization mass spectrometry (ESI-MS)-based assay for the determination of acetylcholinesterase (AChE)-inhibiting activity in snake venom was developed. It allows the direct monitoring of the natural AChE-substrate acetylcholine and the respective product choline. The assay scheme was employed in the screening for neurotoxic activity in fractions of the venom of Bothrops moojeni. AChE inhibition was assessed in two fractions. As a positive control, the established AChE-inhibitor 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW284c51) was used, a dose-response-curve for this compound was generated and the IC_{50}-value for the inhibitor was determined to be (1.60 ± 0.09)10^{-9} mol/L. The dose-response-curve was used as "calibration function" for the venom inhibition activity resulting in equivalent BW284c51-equivalent concentrations of 1.76*10^{-9} mol/L and 1.07*10^{-9} mol/L for the two fractions containing activity. The ESI-MS-based assay scheme was validated using the established Ellman reaction. The data obtained using both methods were found to be in good agreement. The ESI-MS-based assay scheme is therefore an attractive alternative to the standard colorimetric assay.*

7.1 Introduction

The catalytic activity of cholinesterases in the degradation of acetylcholine (ACh) to choline is of central importance in the treatment of Alzheimer's disease (AD). As a general symptom of this disease, reduced cholinergic neurotransmission is observed in the brain of AD patients [1,2]. This deficiency seems to be caused by either reduced activity of choline transferase or by enhanced activity of acetylcholinesterase (AChE) resulting in lower levels of acetylcholine [3 – 5]. Therefore, the predominantly used strategy in the treatment of AD patients is aimed at the potentiation of ACh in the brain by lowering its degradation rate. This is achieved by administering potent inhibitors of AChE, such as tacrine, donezepil, galantamine and rivastigmine. The most significant therapeutic effect is the stabilization or even improvement of the cognitive functions of the patient for at least one year's time [6 – 10]. Since the use of AChE-inhibitors is up to now the most effective therapeutic approach in AD treatment, the demand for alternative inhibitors is high.

Identification of inhibiting activity is performed by assessing the enzymatic activity in presence of the potential inhibitor. The most widely used method for the determination of AChE activity is a colorimetric assay scheme developed by Ellman et al. in 1961 [11]. Since ACh and its product choline do not possess any characteristic spectroscopic properties, an alternative substrate has to be used. To allow the detection by means of light absorption, a secondary reaction is coupled to the enzyme-catalyzed conversion (figure 1b).

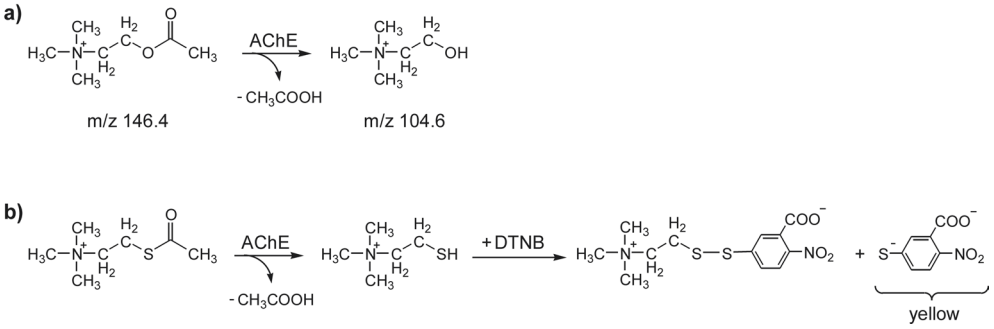

Figure 7.1: Reaction schemes underlying the assays for the determination of AChE activity; a) AChE-catalyzed conversion of acetylcholine to choline; detection by means of ESI-MS on the respective signal traces of m/z 146.4 (AC) and 104.6 (choline); b) AChE-catalyzed conversion of acetylthiocholine to thiocholine; interception of the thiol by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) releases the colored anion, which can be colorimetrically detected.

Acetylthiocholine (ATCh) serves as substrate for AChE and is hydrolyzed yielding the respective thiol compound. This reactive thiol is intercepted by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) releasing the yellow TNB^{2-} anion. The formation of this compound can be monitored by measuring the absorption of the reaction mixture at a wavelength of 412 nm [11].

Mass spectrometry (MS) is a possible alternative to this colorimetric assay scheme. As in MS, analytes are detected depending on their mass-to-charge (m/z) ratio, the spectroscopic properties of the substrates or products are not important. Therefore, MS detection allows the use of naturally occurring substrates without any modifications to the molecular structure or the need of coupling a secondary reaction for detection. In case of the determination of AChE activity, the natural substrate acetylcholine and its product choline are detected at their respective m/z ratios.

A first approach to identify potential inhibitors and to determine their IC_{50} values by means of ESI-MS was presented by Wu et al. in 1997 [12]. Thomas et al. and Lewis et al. developed a MS assay scheme based on matrix assisted laser desorption/ionization from porous silicon surfaces (DIOS) for the monitoring of the AChE-catalyzed hydrolysis of ACh. Using the DIOS-MS assay scheme they also investigated the inhibition of AChE by several known inhibitors [13, 14]. Wall and co-workers employed both, a DIOS-MS and an ESI-MS assay scheme for the determination of kinetic parameters of the ACh hydrolysis mediated by AChE and also studied the inhibition of this reaction by tacrine. The values obtained from both methods were found to be in good agreement [15]. Shen and colleagues presented a high-throughput approach based on the DIOS-MS system for the screening of potential AChE-inhibitors from a compound library reporting a throughput of 4000 samples in a five-hour measurement period [16]. An alternative high-throughput screening approach based on mass spectrometry was presented by Ozbal et al. [17].

Up to now, investigations on potential AChE inhibitors by means of MS were only conducted with pure compounds and not with natural extracts. A promising source for potent AChE-inhibitors are snake venoms. Besides the effects on the haemostatic system, blood coagulation and fibrinolysis, also strong neurotoxic effects, resulting in the immobilization and finally death of the prey, have been reported [18 – 20]. One possible mode of venom action is the selective inhibition of mammalian AChE by, for instance, fasciculins [21].

In order to promote the determination and identification of such anti-AChE agents in snake venoms, an ESI-MS assay scheme was developed, which allowed the direct screening of pre-separated venom fractions for AChE-inhibiting activities in complex biological samples. The assay scheme was employed in the inhibition screening of the venom of *Bothrops moojeni*. The results from the ESI-MS assay were validated with a colorimetric assay based on the Ellman scheme.

7.2 Experimental

Chemicals

The snake venom fractions used in this study were a kind gift from Pentapharm (Basel/Switzerland). Acetylcholinesterase from electric eel (AchE), acetylcholine bromide (ACh), acetylthiocholine iodide (ATCh), 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht/The Netherlands). The p.a. grade ammonium acetate was obtained from Merck (Darmstadt/Germany). All solvents were purchased in LC-MS grade quality from Biosolve (Valkenswaard/The Netherlands).

Sample preparation

All solutions were prepared in buffer (15 mM ammonium acetate/ammonia, pH 7.5 in water) and stored at –18 °C until use. The ACh bromide and ATCh iodide were dissolved in buffer yielding substrate solutions of $1*10^{-4}$ mol/L each. The DTNB was dissolved in buffer to give a $1*10^{-3}$ mol/L solution. The inhibitor BW284c51 was dissolved to give a stock solution with a concentration of $1*10^{-4}$ mol/L, which was diluted to yield solutions of $3*10^{-5}$ mol/L, $1*10^{-5}$ mol/L, $3*10^{-6}$ mol/L, $1*10^{-6}$ mol/L, $3*10^{-7}$ mol/L, $1*10^{-7}$ mol/L, $3*10^{-8}$ mol/L, $1*10^{-8}$ mol/L and $1*10^{-9}$ mol/L, respectively. AChE was dissolved in buffer to yield a solution with a content of 5 μg/mL.

The venom of *Bothrops moojeni* was fractionated by preparative gel permeation chromatography yielding fractions with venom components separated by their molecular masses in solutions. Fractionation of the snake venom was carried out by Anna-Maria Perchuc from Pentapharm Ltd. (Basel/Switzerland). The gel fractions (F28 – F46) were frozen and kept at –18 °C until use.

Instrumental set-up of the FIA/ESI-MS system

For the FIA experiments, a flow-injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/Germany) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL/min of 75/25 (v/v) mixture of methanol and water.

Mass spectrometric detection

For detection, an Esquire 3000$^+$ ion trap mass spectrometer (Bruker Daltonik, Bremen/Germany) equipped with an ESI source was used. All measurements were performed using the positive ion MS mode. Mass spectra were recorded over a range from m/z 100 to m/z 1000 in full scan mode. Instrumental settings are shown in table 7.1. The resulting data was analyzed using DataAnalysis software version 3.1 (Bruker Daltonik, Bremen/Germany).

Table 7.1: List of ESI-MS parameter settings.

Parameter	Setting
nebulizer gas (N_2) pressure	40.0 psi
dry gas (N_2) flow	9.0 L/min
dry gas (N_2) temperature	365 °C
capillary high voltage	5000 V
capillary exit voltage	48.3 V
skimmer voltage	28.9 V
octopole 1 voltage	9.0 V
octopole 2 voltage	1.0 V
octopole amplitude	126.2 Vpp
lens 1 voltage	-2.5 V
lens 2 voltage	-36.6 V
trap drive level	22.5

ESI-MS assay procedure

All ESI-MS-based inhibition assays were performed according to the following general procedure: AChE solution (50 μL) and 50 μL of the respective venom fraction solution were combined with 300 μL buffer and pre-incubated at RT for 10 minutes. The reaction was started by adding 400 μL of the substrate ACh solution to the mixture. Aliquots of 50 μL were sampled from the reaction mixture every 2 minutes for a complete time span of 20 minutes. The aliquots were quenched in 950 μL methanol to stop effectively the conversion by rapid denaturing of the catalytic enzyme. The quenched samples were subsequently injected into the FIA/ESI-MS system and analyzed in triplicate. The injection volume was set to 5 μL.

For the reference assays without inhibition, 50 μL of buffer were added to the enzyme instead of the venom fraction solution. In case of the BW284c51 assays, 50 μL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from $6.25*10^{-7}$ mol/L to $6.25*10^{-11}$ mol/L.

UV/vis assay procedure

In order to allow the direct comparison between ESI-MS assays and UV/vis assays, the reaction conditions and ratios of reagents had to be similar. Therefore, the colorimetric inhibition assays were carried out according to the following scheme: 300 μL of the AChE solution, 300 μL of venom fraction solution and 160 μL of the DTNB solution were mixed, diluted with 1640 μL buffer and pre-incubated at RT for 10 minutes in order to allow the DTNB to

react with possibly present thiol functions in the protein content of this mixture. 100 μL of this mixture was transferred to the wells of a 96-well microtitation plate. The reaction was started by adding 100 μL of the substrate ATCh solution to each well. The reaction progress was monitored by measuring the increase of absorption at 412 nm wavelengths on a SpectraMax 250 plate reader (Molecular Devices, Munich/Germany).

For the reference assays without inhibition, 50 μL of buffer were added to the enzyme instead of the venom fraction solution. In case of the BW284c51 assays, 50 μL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from $6.25*10^{-6}$ mol/L to $6.25*10^{-11}$ mol/L.

7.3 Results and discussion

7.3.1 Properties of the AChE-inhibition assays

For the investigation of inhibitor activity towards AChE in the venom of *Bothrops moojeni*, the venom fractions are assayed individually against AChE and the natural substrate ACh. In the course of the reaction, ACh is hydrolyzed by AChE to choline and acetic acid (figure 7.1a). This is monitored by ESI-MS. Typical mass spectra obtained from quenched samples at the beginning of the reaction and at the end are shown in figure 7.2.

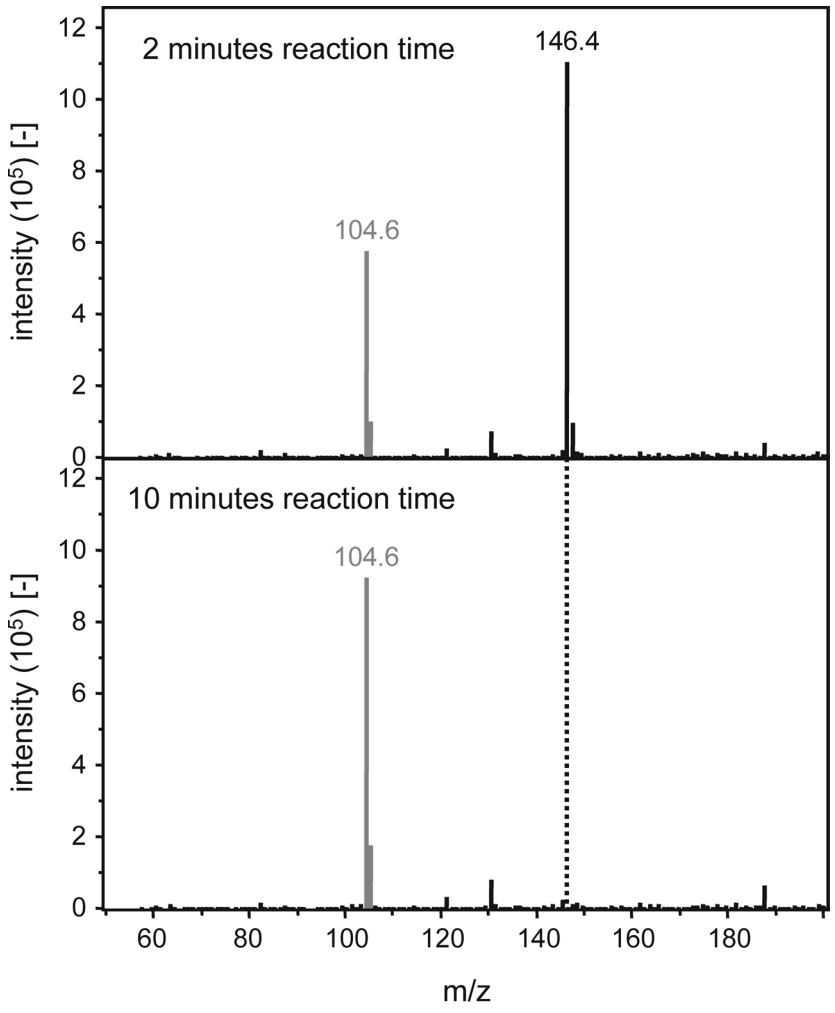

Figure 7.2: Mass spectra obtained from samples of the reaction mixture after 2 minutes and after 10 minutes reaction time. The mass spectrum after 2 minutes reaction time shows signals for both, the substrate compound AC (m/z 146.4) and the product choline (mz 104.6). After 10 minutes reaction time, only the signal for choline is observed, while the substrate signal is completely decreased (position of AC signal indicated by the dotted line).

After 2 minutes reaction time, signals for both substrate and product are observed. After a reaction time of 10 minutes, the signal for ACh is not longer detectable and the product signal increased significantly. Therefore, it can be concluded that the conversion of the substrate is completed.

Subsequent injections of the quenched samples allow to monitor the progress of the enzyme-catalyzed hydrolysis.

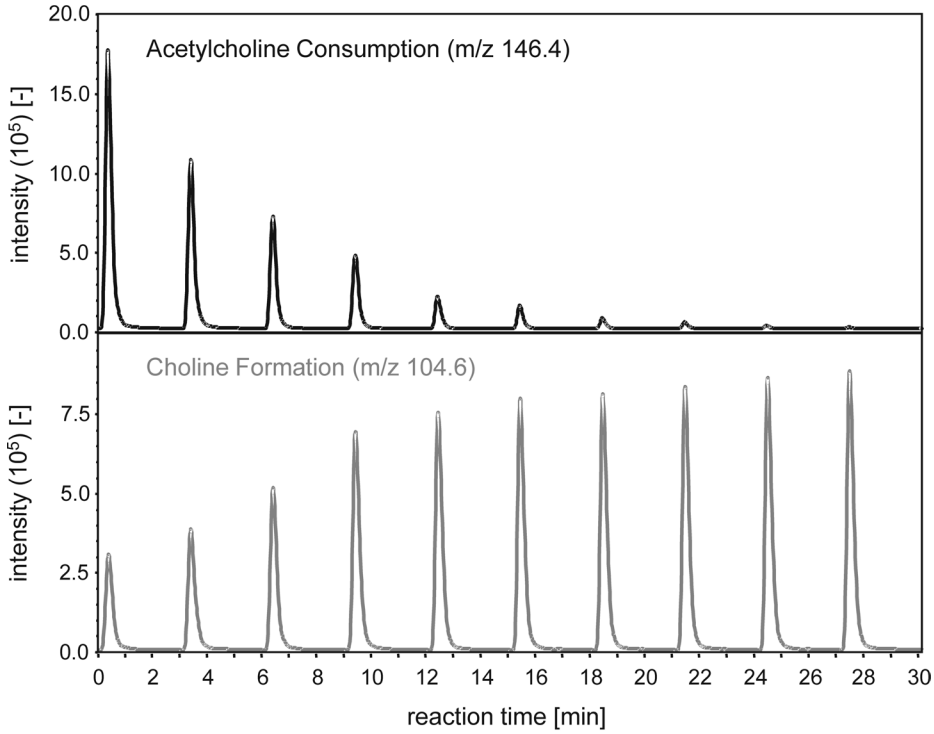

Figure 7.3: Injection profile recorded on the signal traces of substrate and product showing clearly the decrease in AC concentration and the increase of choline concentration in the reaction mixture over the course of time.

Figure 7.3 shows the signal traces for the substrate and the product. The consumption of the ACh and the formation of choline are directly observed by the decrease and increase of the peaks on the respective m/z-traces. Integration of the peak areas gives a measure of the concentration the respective compounds present at a given time.

The relative reaction progress (RRP) at a time was determined on the basis of using the substrate as internal standard for the product, as discussed in chapter 6. The value for the RRP ranges from 0% (no product formed) to

100% (complete conversion of the substrate). Plotting the RRP values versus the reaction time for each assay results in a full time-resolved reaction profile.

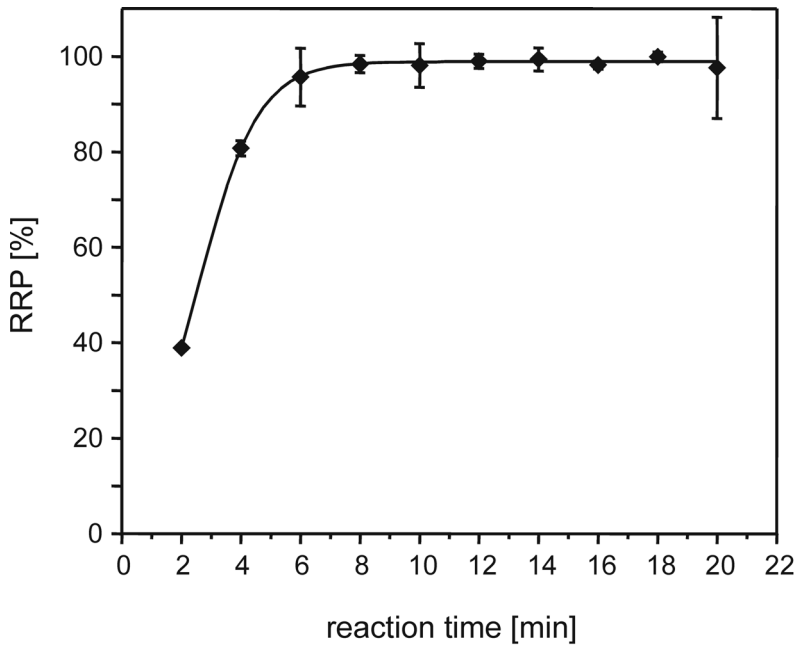

Figure 7.4: Complete reaction profile showing the relative reaction progress at different time points; derived from the ESI-MS assay scheme of the reference assay without any inhibitor present. The relative standard deviation ranges from 1.3% to 10.9% (n = 3)..

The profile depicted was derived from the reference assay without any inhibitor present. The profile shows a typical course of an enzyme-catalyzed reaction with a high initial reaction rate. The conversion of the substrate seems to be completed after 8 minutes reaction time.

7.3.2 Assessment of inhibitory activity

In order to assess inhibitor activity in the snake venom, the RRP profiles derived from the assays in presence of each venom fraction were compared

to the reference profile. This can be investigated closer using a correlation plot (figure 7.5a).

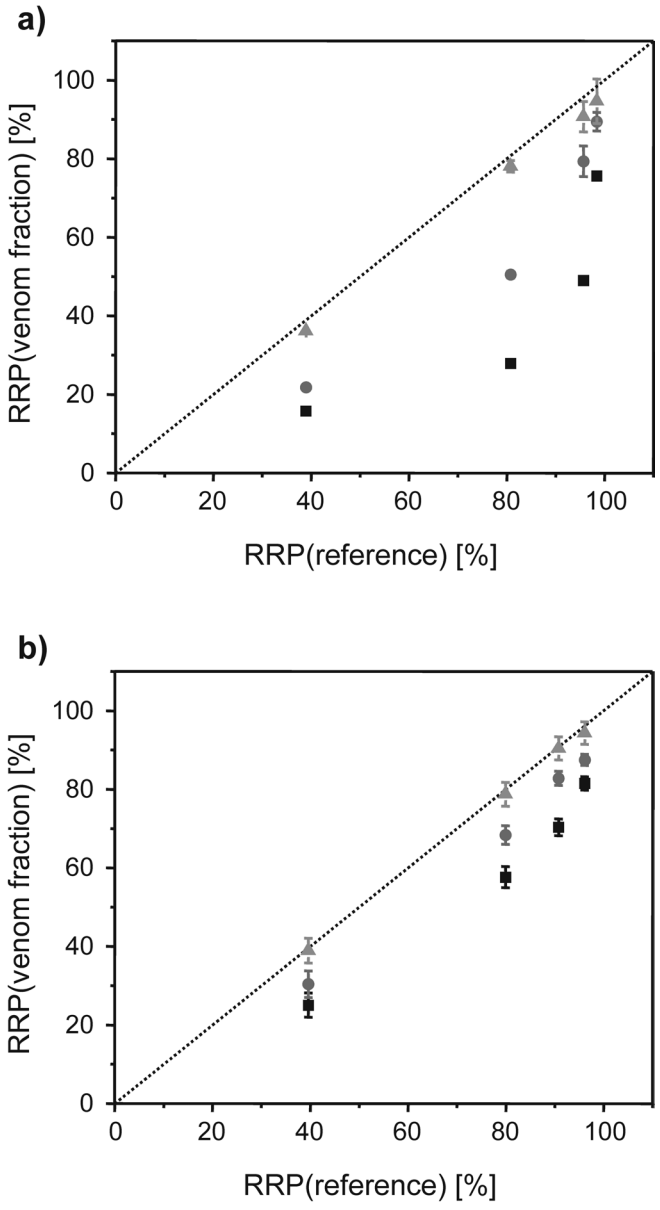

Figure 7.5: Correlation plots derived from a) the ESI-MS assays and b) from the colorimetric assays. The relative reaction progress during 8 minutes reaction time in the presence of the venom fractions F34 (■), F33 (●) and F29 (▲) is plotted versus the relative reaction progress of the reference assays. The dotted line indicates the position for complete correlation of reference and screening assays.

In this figure, the RRP values of the reactions in presence of the venom fractions F33 and F34 are plotted versus the RRP values of the reference assay. Close correlation (no change in catalytic activity of AChE) is indicated by points close to the dotted line. The correlation plots of the assays in presence of F33 and F34 show significant divergences from the line, thus indicating a loss in enzymatic activity in the respective assays. This leads to the conclusion that in those fractions an inhibitor for AChE must be present. The correlation plots of the assays in presence of the other venom fractions (shown as example for F29) did not exhibit any significant divergences. Therefore, no inhibitory activity is assigned to be present in those fractions.

7.3.3 Control experiments by colorimetric assays

To verify this finding, control assays following a modified Ellman procedure were performed. The relative reaction progress in percent at a given time in the colorimetric assay was calculated from the ratio between actual absorbance and the maximum absorbance (after completion of the conversion). Plotting the RRP values of the individual assays versus the values obtained from the reference assay results in a correlation plot similar to the one derived from the MS assays (figure 7.5b).

As assessed from the ESI-MS-based assays, a significant divergence from the reference assay is observed indicating the slower reaction progress in the presence of F33 and F34. Also, no significant divergences could be observed in the correlation of the other fractions assays. This supports the conclusion drawn from the ESI-MS assays that only in fractions F33 and F34, an AChE-inhibiting compound is present.

7.3.4 Comparison of venom inhibition potential with a known inhibitor

In order to further characterize the inhibition potential, the inhibitory activity found in the fractions was related to that of the known inhibitor BW284C51. Therefore, dose-response profiles for the inhibition of AChE by BW284C51 were generated employing both, the ESI-MS- and the UV/vis-absorption-based assay schemes. Assays were performed in the presence of varying concentrations of inhibitor and the respective RRP values were calculated as described above. The RRP values of the inhibition assays after 6 minutes reaction time were related to the RRP value found in the reference assay at the same time point (100% assay performance) and plotted versus the concentration of BW284C51 (see figure 7.4).

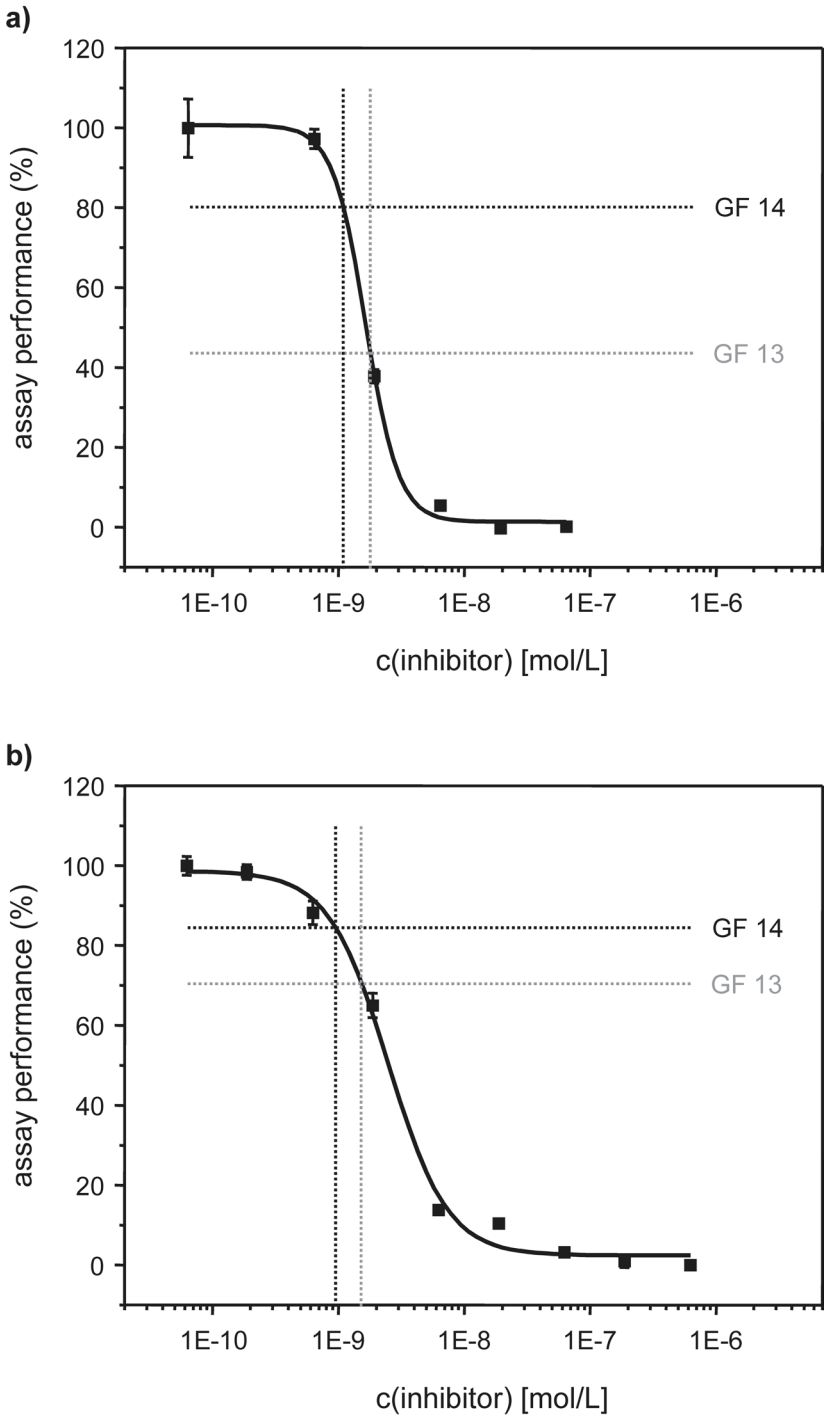

Figure 7.4: Dose-response plots for the AChE inhibitor BW284C51 derived from a) ESI-MS assays and b) colorimetric assays; the respective assay performance in the presence of F34 and F33 are indicated by the horizontal dashed lines; the equivalent concentrations of BW284C51 are given by the intersections with the response curve.

From these plots, the IC_{50}-values of BW284C51 towards AChE were determined to be (1.60 ± 0.09)*10^{-9} mol/L (MS assay) and (2.48 ± 0.24)*10^{-9} mol/L (UV/vis-absorbance assay), respectively.

The dose-response profiles from both assay schemes were used to relate the inhibitory activity in the venom fractions F33 and F34 to the inhibitory activity of BW284C51. Therefore, the assay performance of the respective reactions after 6 minutes reaction time was determined and the corresponding theoretical concentrations of BW284C51 were calculated (table 7.2).

Table 7.2: Equivalent concentrations of BW284C51 calculated for the inhibition activities found in F33 and F34.

Venom Fraction	**Equivalent Concentration of BW284C51**	
	ESI-MS	UV/vis-absorption
F 34	1.76*10^{-9} mol/L	1.42*10^{-9} mol/L
F 33	1.07*10^{-9} mol/L	0.89*10^{-9} mol/L

Considering the fact that two different assay schemes were employed, one based on the direct detection of product and substrate and the other employing a secondary reaction for the generation of a detectable product, the values calculated for the IC_{50} of BW284C51 and for the equivalent concentrations for the venom fractions correlate well.

7.4 Conclusions

An ESI-MS-based assay was developed for the rapid screening for AChE-inhibitory activities in complex biological samples. The assay was applied successfully to the inhibition screening of snake venom. The inhibitory activity found in the venom fractions was quantified in relation to the known AChE inhibitor BW284C51 using its dose-response plots as calibration curve, thus giving an independent measure for comparison of inhibition activity in different fractions. All findings resulting from the ESI-MS assay scheme were controlled by colorimetric assays, thus validating the ESI-MS methodology. The results were found to be in good agreement with each other.

To sum up, ESI-MS is an excellent alternative detection method for the monitoring of enzyme-catalyzed assays. The major advantage over the optical methods is the possibility to monitor the naturally occurring substrate ACh and its product choline, which are not detectable by any direct optical means. The ESI-MS assay scheme proved to be generally applicable not only to the investigation of pure substances, but also for highly complex biological samples. Since ESI-MS assays offer the opportunity to monitor several enzymatic conversions in parallel, further work will be directed to the development of a multiplexed inhibition assay scheme

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Chapter 8

Matrix Effects in the ESI-MS-Based Monitoring of Enzymatic Bioassays[‡]

High Turbulence Liquid Chromatography (HTLC) is presented as a means to reduce ion suppression in simultaneous multianalyte mass spectrometric bioassays. In this study, the effects of enzymes present in the sample on the signal response of five analytes were simultaneously investigated over a protein content range from 0 μg/mL to 38 μg/mL by means of direct flow-injection mass spectrometry. As model enzymes, trypsin, thrombin and chymotrypsin were selected. Without HTLC, the matrix effects caused both signal suppression and signal enhancement depending on the nature of the analyte and the concentration of matrix in the sample and were found to be generally intolerably large. The deviation from the mean signal response as a measure of distortion was found to be between 14% up to 112%. The addition of an excess of methanol as means of sample clean-up was investigated and found not to be sufficient. By employing high turbulence flow chromatography for on-line sample preparation, it was possible to reduce the matrix effects to a minimum for all model systems investigated. In case of trypsin the distortion could be lowered from 41.9% to 2.6%.

[‡] A. Liesener, U. Karst, accepted for publication in *J. Sep. Sci.* **2005**.

8.1 Introduction

A disadvantage of the use of ESI-MS for the detection in enzymatic bioassays is the possible occurrence of matrix effects during the ionization process, since the quantification of the analytes (product and unreacted substrate compounds) has to be performed in presence of the converting enzyme. It is already known from literature that matrix components such as proteins may have a large influence on the efficiency of the ESI process [1 – 4], and may thus lead to either ion suppression or enhancement. A chromatographic separation of the sample prior to the ionization can help reducing these effects, but the chromatographic parameters have to be carefully evaluated in order to prevent undesired coelution of the analytes and matrix constituents [5, 6]. The use of conventional reversed-phase liquid chromatographic minibore columns may not be feasible in all cases, since the repeated direct injection of the protein-containing sample might result in clogging or damage of the stationary phase, thus leading to a strongly reduced column lifetime. Therefore, a sample preparation procedure, in which the disturbing enzyme is efficiently removed, is required. Due to the large numbers of samples generated by the time-resolved conversion monitoring experiments, a fast and automated sample clean-up method would be most helpful.

A sample preparation method fulfilling these criteria is high turbulence liquid chromatography (HTLC). In this approach, the samples are directly injected into a high flow of aqueous mobile phase stream and transferred onto a microbore column containing a large particle size stationary phase, where the low molecular weight analytes are retained, while the high molecular weight

compounds and salts are rapidly washed off. In a second step, the analytes are eluted from the column to the mass spectrometer utilizing an organic mobile phase. This technique was introduced in 1997 by Quinn and Takarewski [7] and its potential as fast on-line sample preparation method was soon recognized by several groups [8 – 10]. Zimmer et al. compared the performance of the HTLC method with established automated solid-phase extraction and liquid-liquid extraction procedures, finding even better results for HTLC [11]. Today, HTLC is widely used as tool for on-line sample clean up in combination with direct injection high-throughput bioanalysis [12, 13]. Fields of application are not only pharmacokinetic studies in pharmaceutical research, but successful applications in therapeutic drug monitoring of immunosuppressants [14] and in the determination of trace level pesticides in water [15, 16] have been described as well.

To investigate the extent of matrix effects on the ESI-MS-based analysis of multianalyte enzymatic conversions, the resulting signal response of several model samples analyzed by means of direct flow-injection analysis (FIA) is evaluated. The reduction of those effects by employing HTLC as means of on-line sample preparation is assessed by comparative experiments.

8.2 Experimental

Chemicals

The substrates used in this work were a kind gift from Pentapharm Ltd. (Basel/Switzerland). Enzymes were obtained in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht/The Netherlands). All solvents were purchased in LC-MS grade quality from Biosolve (Valkenswaard/The Netherlands).

Sample preparation

To investigate the matrix effects, which might occur in the monitoring of enzymatic conversion by means of ESI-MS, typical samples were prepared. As model matrix compounds, three proteases (trypsin, thrombin and chymotrypsin) were selected. For each enzyme, a solution was prepared containing 1 mg protein per 1 mL buffer (15 mmol/L ammonium acetate, pH 7.5). The substrates were dissolved in buffer yielding a stock solution with concentrations of $5*10^{-5}$ mol/L of each compound. 1400 μL of this solution were combined with 100 μL thrombin solution containing 10 μg of the enzyme. The reaction mixture was left overnight at room temperature to ensure a complete conversion of the substrates to their respective products, which serve as analytes in the study.

Table 8.1: List of analytes and the signal traces used for detection

Analyte	Analyte Structure	$[Analyte+H]^+$ Signal Trace
XII	H-D-CHT-Gly-Arg-OH	m/z 385
IX	H-D-Leu-PHG-Arg-OH	m/z 421
X	CH_3OCO-D-CHA-Gly-Arg-OH	m/z 443
VII	CH_3SO_2-D-CHA-Abu-Arg-OH	m/z 491
PC	H-D-Lys(Cbo)-Pro-Arg-OH	m/z 534

Aliquots of 20 μL of this solution were mixed with various volumes of model enzyme solution (0 μL, 10 μL, 20 μL, 40 μL, 80 μL, 180 μL and 380 μL) and filled up with buffer to a total volume of 400 μL each. Each mixture was then diluted with 450 μL of buffer or methanol. The dilution with methanol simulates the quenching of an ongoing reaction by means of a rapid denaturing of the active enzyme. Finally, each sample contained the five analytes in a concentration of $2.3*10^{-7}$ mol/L and a defined amount of enzyme (0 μg, 1 μg, 2 μg, 4 μg, 8 μg, 18 μg and 38 μg, respectively). Each sample was measured fivefold, and the mean value of the resulting peak areas on each signal trace was calculated.

Instrumental set-up FIA experiments

For the FIA experiments, a flow-injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/Germany) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL/min of pure acetonitrile (ACN).

Instrumental set-up HTLC experiments

For the HTLC experiments, a Cyclone TurboFlow column of 0.5 mm * 50 mm dimension with 60 μm particle size and 100 Å pore size (Cohesive Technologies, Milton Keynes/UK) was selected. A schematic overview of the experimental set-up for the HTLC experiments is shown in 8.1.

The core of the set-up is a two-position six-port valve (integrated into the mass spectrometer) used for direction of the mobile phase streams. In the first step of each experiment, 5 µL of the respective sample is injected by an autosampler (HP1100 model G1313A, Agilent, Waldbronn/Germany) into the loading stream (pure water at 1.2 mL/min) delivered from a Shimadzu LC10ADvp HPLC pump (Duisburg/Germany) and transferred onto the extraction column. The analytes are retained on the stationary phase, while the matrix components are flushed to waste for 60 s. The mobile phase for elution (pure ACN at 0.3 mL/min), delivered by a HP1100 model G1312A HPLC pump (Agilent) is flowing directly to the mass spectrometer (Loading Step). After valve switching, the mobile phase is directed onto the column, and the analytes are back-flushed from the stationary phase into the ionization source for a duration of 60 s (Elution Step). In the third step, the aqueous phase is again directed to the extraction column by switching of the valve, equilibrating and reconditioning the stationary phase for 30 s (Conditioning Step). The complete cycle time was 2.5 min per sample.

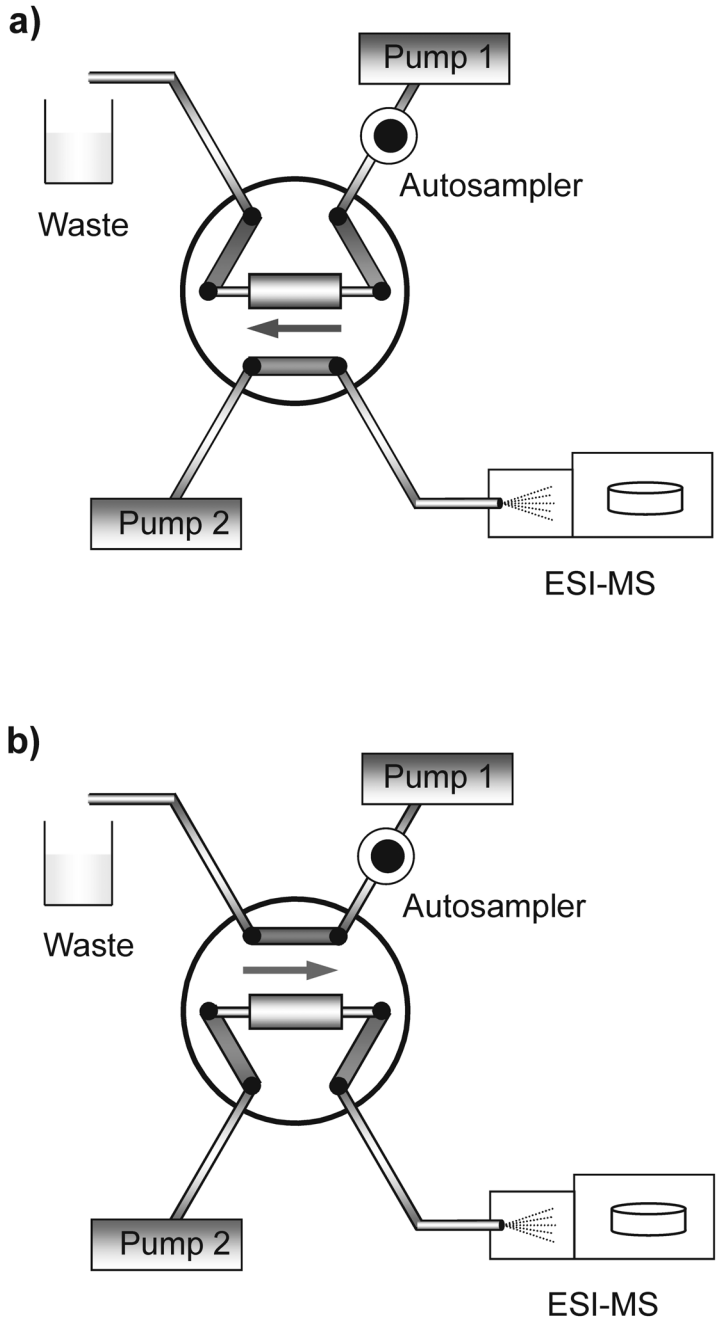


Figure 8.1: Schematic overview of the HTLC experimental set-up. a) loading position of switching valve: sample is injected into the aqueous stream from pump 1; analytes are trapped on the HTLC column; matrix constituents are washed to waste. b) elution position of switching valve: flow direction through the HTLC column is inverted; ACN elution stream from pump 2 flushes analytes from the column to the MS detector.

Mass spectrometric detection

For detection, an Esquire 3000$^+$ ion trap mass spectrometer (Bruker Daltonics, Bremen/Germany) equipped with an ESI source was used. All measurements were performed using the positive ion MS mode. Instrumental settings are shown in table 8.2. The resulting data was analyzed using DataAnalysis software version 3.1 (Bruker Daltonics, Bremen/Germany).

Table 8.2: List of the ESI-MS parameter settings.

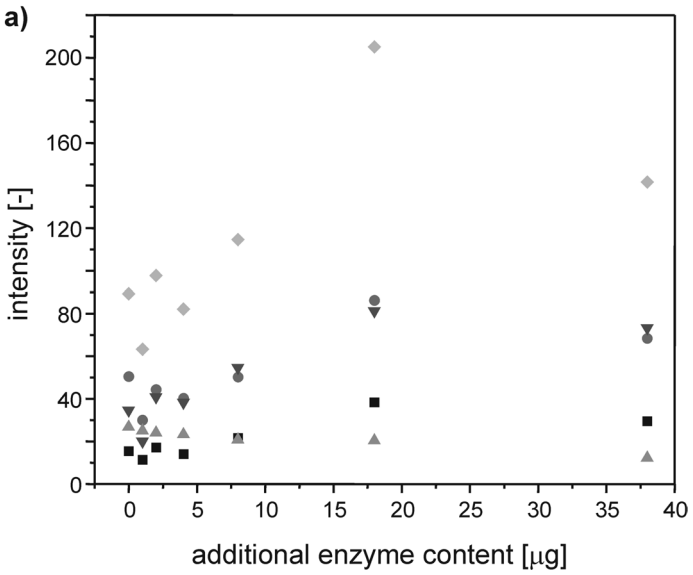
Parameter	Setting
nebulizer gas (N_2) pressure	40.0 psi
dry gas (N_2) flow	10.0 L/min
dry gas (N_2) temperature	365 °C
capillary high voltage	5000 V
capillary exit voltage	156.6 V
skimmer voltage	15.0 V
octopole 1 voltage	11.69 V
octopole 2 voltage	3.26 V
octopole amplitude	127.9 Vpp
lens 1 voltage	-6.1 V
lens 2 voltage	-49.8 V
trap drive level	57.0

8.3 Results and discussion

In order to investigate the influence of enzyme content present in a typical model sample, four sets of experiments were conducted and compared.

8.3.1 FIA experiments

For the first set of measurements, the enzyme-spiked samples were directly analyzed by FIA-MS. The signal intensity was plotted versus the enzyme content added, resulting in response profiles of the analytes for each model matrix (see figure 8.2).



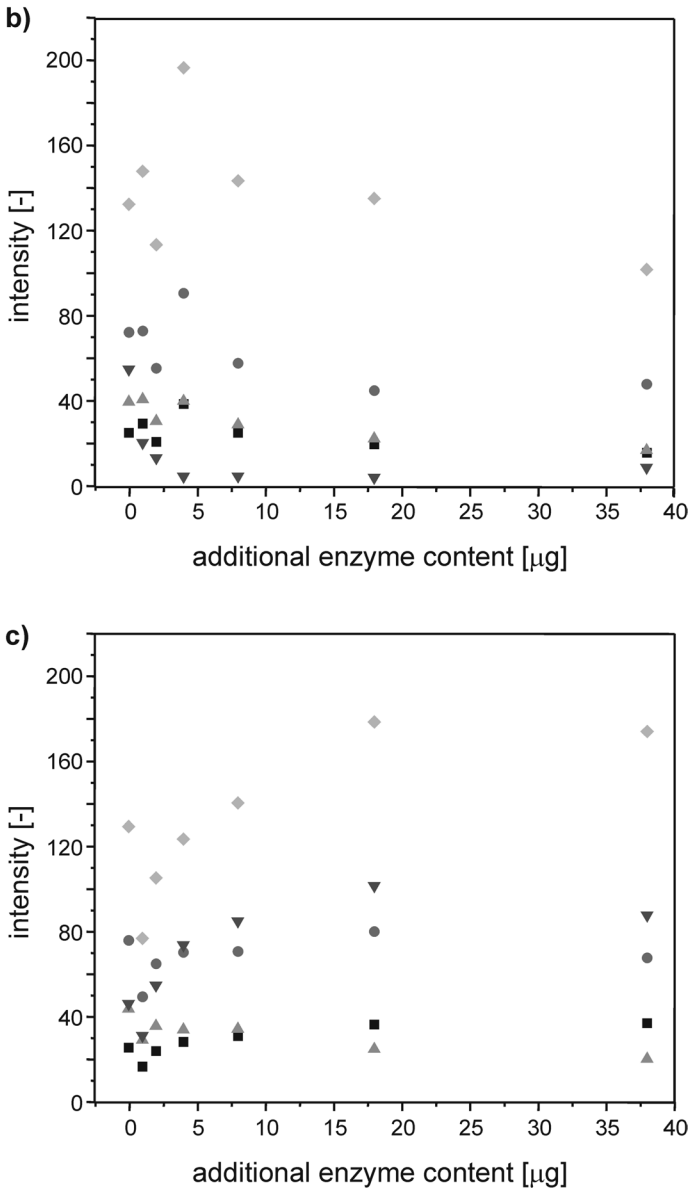


Figure 8.2: Signal response profiles for the addition of trypsin (a), thrombin (b) and chymotrypsin (c) showing the influence of matrix components on signal intensity of the analytes (■ XII, ● IX, ▲ X, ▼ VII and ◆ PC). Samples were analyzed using the FIA method with a carrier stream of 0.3 mL/min pure ACN.

The profiles in each case clearly show the large matrix effects on the respective signal intensities. In case of trypsin addition (figure 2a), the profiles for VII, IX, XII and PC all exhibit a similar behavior. An initial drop in signal intensity is followed by an increase and another slight drop in the response. A second significant increase of the intensities is observed with higher matrix contents reaching maximum values at the addition of 18 μg of enzyme. Another decrease of the intensities can be assessed at the highest enzyme concentration. The signal intensity of X however decreases steadily with increasing amount of matrix trypsin content.

With the addition of thrombin (figure 8.2b) the observed effects are different. The signal responses for IX, X, XII and PC are now similar with an initial slight increase, followed by a drop in the response in the presence of 2 μg of the enzyme, a maximum response at the addition of 5 μg and a steady decrease of the intensity with higher matrix concentration. However, in the presence of increasing amounts of thrombin, the signal response of VII is rapidly decreasing.

The effect of the chymotrypsin addition (figure 8.2c) is similar for all five signal traces, characterized by at first a drop in the response and an increase in the intensities reaching a maximum value with a enzyme addition of 18 μg followed by a slight decrease in intensities at the highest enzyme concentration. The three response profiles clearly show the difference in matrix effects depending on the matrix composition (each model protease generating different profiles) but also on the analyte, since the presence of one enzyme causes different trends in the response profiles for different analytes.

In the second set of experiments, the matrix effects of the model enzymes on the signal response in the presence of methanol was investigated. Therefore, the methanol-diluted samples were analyzed by direct FIA-MS. The response profile for the addition of thrombin is shown as example in figure 8.3.

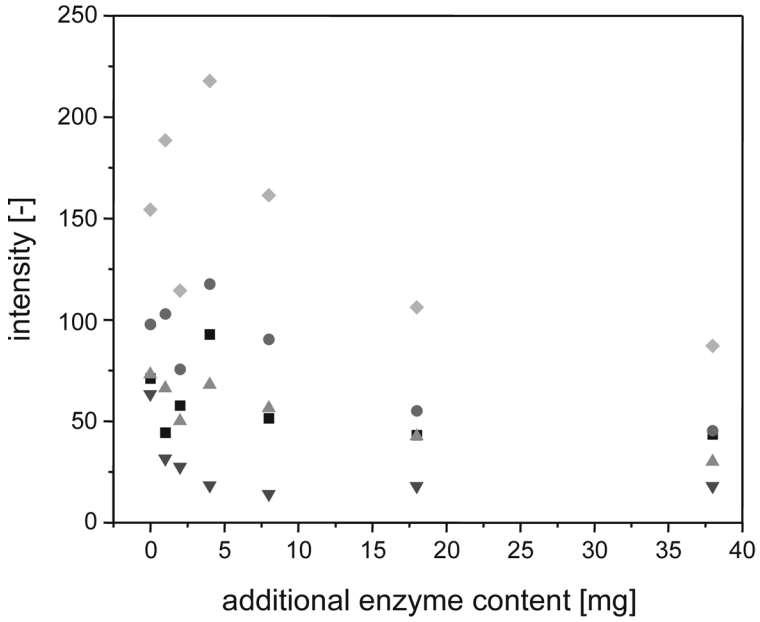


Figure 8.3: Signal response profile for the addition of thrombin and subsequent dilution with methanol (■ XII, ● IX, ▲ X, ▼ VII and ◆ PC). Samples were analyzed using the FIA method with a 0.3 mL/min carrier stream of pure ACN.

Comparing the FIA response profiles of the buffer- and the methanol-diluted samples with addition of thrombin indicates that the presence of a large excess of the organic solvent does not lead to an elimination of the matrix effects. However, the signal intensity of each analyte is higher when methanol is used for dilution. The same holds for the response profiles of the trypsin- and chymotrypsin-containing samples. This might be due to a more efficient electrospray ionization process in the presence of methanol.

8.3.2 HTLC experiments

To study the effectiveness of HTLC for minimizing the matrix effects on the signal responses of the selected analytes, the samples diluted with buffer or methanol were injected as described above. A respective chromatogram showing the five signal traces is presented in figure 8.4.

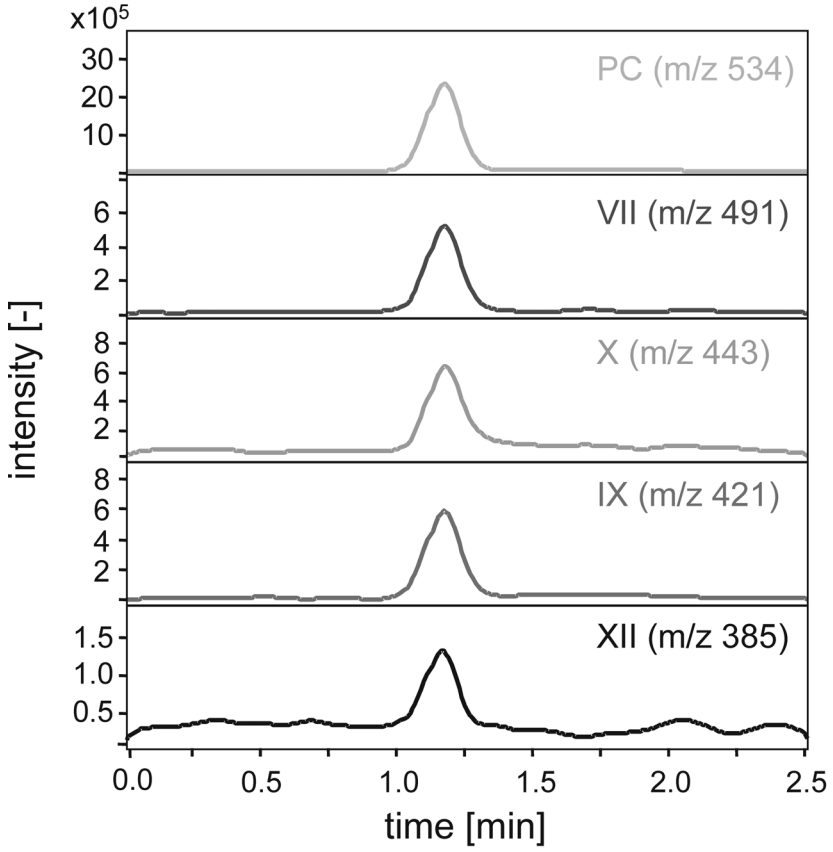


Figure 8.4: Typical chromatogram obtained by means of HTLC sample clean-up procedure. Depicted are the five signal traces for the respective analytes. The sample analyzed contained 38 μg of trypsin; loading was performed on a HTLC extraction column using pure water (1.2 mL/min), and backflush-mode elution was performed by switching to pure ACN (0.3 mL/min).

All analytes are eluted simultaneously from the column. The total run time is identical to the FIA method. A comparison of the signal trace of PC in the presence of 38 μg trypsin (figure 8.5) shows a significantly improved peak shape when employing HTLC.

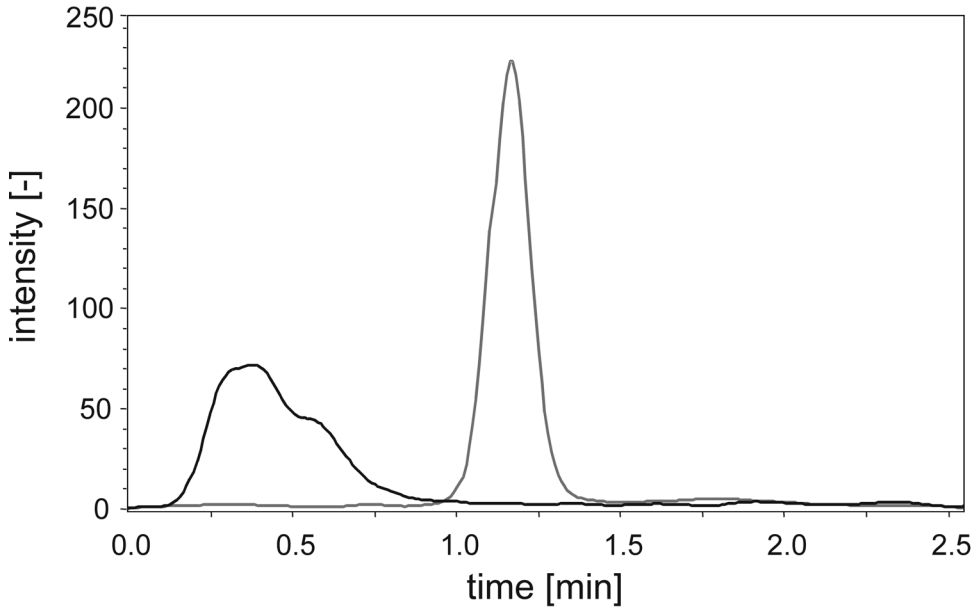


Figure 8.5: Chromatograms from a sample containing 38 μg trypsin (signal trace of PC); obtained by FIA (—) and by HTLC (—); analytes were ionized in ACN stream (0.3 mL/min).

Due to the operation in the backflush mode, the analytes are trapped and focused on top of the column and eluted directly backwards without further band broadening. The peak width was reduced to 30 s compared to the FIA peak width of 1 min. The tailing is significantly reduced leading to an almost Gaussian peak shape. The total peak height is substantially increased, thus resulting in a better signal-to-noise ratio.

8.3.3 Comparison of FIA and HTLC experiments

Each measurement was carried out in fivefold and the respective mean intensity values for each signal trace were calculated. The relative standard deviation for each measurement describes the precision of the experiment. To compare the response profiles generated by the FIA and HTLC experiments, the mean response values over a complete signal response profile and the standard deviation for the respective signal traces were calculated. Thus, the relative standard deviation from the mean response value can be considered to be the distortion of the signal response, thus being a measure for the extent of the matrix effect of the model enzyme on the respective analyte. The higher the value, the higher is the influence of the matrix effect.

Figure 8.6 shows a comparison of the response profiles of PC in the presence of trypsin obtained from FIA and HTLC experiments. The calculated mean intensity value for each set of measurements and the respective standard deviation is indicated by the dotted lines. The comparison clearly shows the much better accuracy of the HTLC response profile with an accuracy of +/- 2.6%. The accuracy of the FIA profile is significantly worse with +/- 41.9% deviation. Additionally, the total signal intensity could also be improved by a factor of 1.8 by employing HTLC.

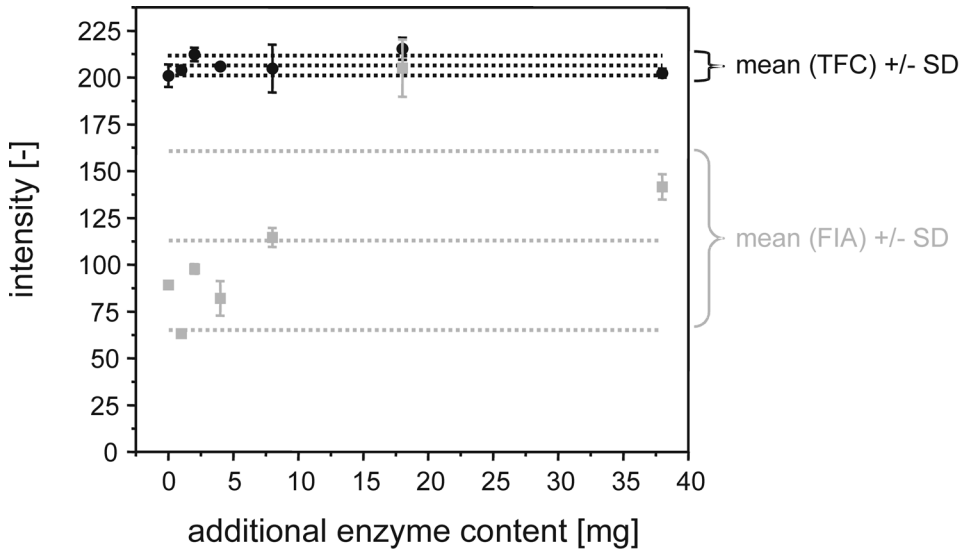


Figure 8.6: Comparison of signal response profiles from FIA and HTLC experiments for analyte PC in presence of trypsin; analytes were ionized in ACN stream (0.3 mL/min). The dotted lines represent the calculated mean values for the signal response for both methods and the standard deviation intervals as measure for the distortion of the signal intensity by the matrix effect. The error bars on each data point indicate the precision of the respective fivefold experiment.

The results of the different experiments with respect to the total mean signal intensity, accuracy and mean precision are provided in table 8.3.

In almost all cases, a significantly lower value for the distortion of the signal by the matrix constituents could be achieved when employing HTLC. The dilution of the sample with methanol gave higher response values in all FIA experiments, but did in most cases not reduce the distortion significantly. The methanol-diluted samples in the HTLC experiments did not provide higher signal responses except for analyte XII.

Table 8.3: Summary of calculated mean signal response values, distortion and mean precision of each set of experiments.

		Trypsin				Thrombin				Chymotrypsin			
		FIA		TFC		FIA		TFC		FIA		TFC	
		Buffer	MeOH	Buffer	MeOH	Buffer	MeOH	Buffer	MeOH	Buffer	MeOH	Buffer	MeOH
XII	mean (10^6)	2.1	4.3	0.9	2.1	2.5	5.8	1.2	2.9	2.8	5.5	1.0	2.2
	distortion (%)	45.8	19.1	9.0	28.6	29.4	32.0	6.4	32.3	25.1	10.8	24.4	26.0
	precision (%)	9.2	8.9	10.0	25.5	6.2	5.4	6.8	18.0	5.8	9.0	11.3	18.0
IX	mean (10^6)	5.3	7.8	5.9	3.9	6.4	8.3	5.2	4.3	6.9	8.6	5.3	4.3
	distortion (%)	35.6	27.1	5.5	8.6	25.6	31.4	7.3	10.0	14.2	22.6	17.1	8.6
	precision (%)	4.0	6.0	5.1	5.5	2.9	4.0	5.8	5.1	3.8	6.2	6.2	5.1
X	mean (10^6)	2.2	3.2	6.8	6.6	3.2	5.5	5.7	5.8	3.2	4.7	6.3	6.1
	distortion (%)	21.8	27.1	8.1	13.1	29.7	28.0	5.9	7.2	24.2	27.4	18.1	10.9
	precision (%)	9.9	10.0	5.7	4.5	5.5	6.0	5.1	4.5	7.2	9.5	7.6	5.6
VII	mean (10^6)	4.9	7.9	4.8	4.3	1.6	2.7	2.4	3.5	6.9	8.9	4.3	5.0
	distortion (%)	44.8	37.2	5.3	8.1	112.3	62.8	9.8	8.3	36.8	29.7	10.4	8.6
	precision (%)	10.2	9.6	4.6	5.7	6.6	5.9	6.8	5.8	4.7	5.6	7.4	6.3
PC	mean (10^6)	11.3	16.2	20.7	19.5	13.9	14.7	12.1	11.8	13.3	17.1	18.9	21.9
	distortion (%)	41.9	43.8	2.6	2.5	21.8	32.0	5.2	6.5	27.2	26.7	12.2	7.7
	precision (%)	5.3	4.6	2.3	3.7	3.1	3.9	4.2	5.2	4.1	6.8	5.5	3.3

However, the precision of the quantification of XII under HTLC conditions in presence of methanol is rather poor, which might be caused by an inefficient trapping of this compound on the column in presence of an organic solvent. Generally, the precision of the quantification for both different methods, FIA and HTLC, is comparably good and ranges from 2.3% to 10.2%. There is also no difference between buffer- and methanol-diluted samples.

8.4 Conclusions

The observed matrix effects on the signal intensity of the respective analytes are intolerably large under FIA conditions. The influence of the matrix components can be either ion suppression resulting in a loss of signal intensity or ionization enhancement leading to higher response values. These effects seem to be not only dependent on the nature but even more on the amount of matrix component present. Thus, it is not possible to predict the matrix effects on analyte responses, especially when working with complex, unknown matrices. A simple dilution of the samples with methanol did not lead to a satisfying reduction of the disturbing effects. With the use of HTLC as means of on-line sample preparation, the distortion by matrix effects was strongly reduced. An additional effect of employing HTLC in the backflush mode is the improvement of peak shapes, thus leading to better sensitivity of the assay. Furthermore, removing involatile matrix components by means of HTLC could also significantly reduce the contamination of the ion source, thereby reducing the maintenance cycles required. The instrumental set-up of HTLC can be readily realized with equipment, which is already available in

most analytical laboratories making this sample preparation technique comparatively cost-effective.

A drawback of the HTLC method is the limited loading capacity of the extraction column, which requires a careful adaption of the amount of analyte to be extracted. The extraction columns do have a limited lifetime, which can be prolonged by intense flushing with ACN. However, the columns used in this study showed no significant loss in performance even after 1000 sample injections. Therefore, it can be concluded, that HTLC is a highly valuable and easy-to-use tool for analyzing enzymatic conversions by means of ESI-MS.

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Chapter 9

Concluding Remarks and Future Perspectives

A rapid and efficient assay scheme for the screening of relevant activities in highly complex biological samples was introduced in this thesis. The detection method of choice was electrospray ionization mass spectrometry (ESI-MS).

The general applicability of MS as means of detection in bioassays was successfully demonstrated on several enzymatic systems, thus proving the versatility of the method. The results obtained from MS-based assays were in all cases compared to those from independent reference methods in order to validate the approaches. A high degree of compliance was found for the assay schemes.

The most important advantage of mass spectrometric detection in comparison with the standard optical or radiometric detection methods is its general suitability for multiplexed analyses. This was demonstrated by the successful simultaneous quantification of two enzymes in one sample and the development of an assay scheme, which allowed to assess enzymatic activities towards seven different substrates in parallel. On the basis of this approach, a multiplex assay for the proteolytic activity screening in snake venom was introduced. This methodology allowed the rapid characterization

and identification of the respective enzymatic activities in the single fractions of the pre-separated venom. This type of activity assessment is a highly promising approach towards a general high-throughput screening procedure, which allows the fast identification of lead activities in complex samples.

Furthermore, an assay for determination of acetylcholinesterase inhibition was developed. This assay scheme makes use of the capability of mass spectrometry to detect the natural substrate acetylcholine and its product choline. These compounds are not amenable to the detection by any optical detection means, thus highlighting the value of mass spectrometric detection, which allows studying enzymatic reactions with natural substrates. By employing this MS-based assay, it was possible to identify and quantify the inhibitory potential of the snake venom towards acetylcholinesterase, thus demonstrating the ability of the methodology to screen not only for enzymatic activities, but also for putative inhibitors in complex biological extracts.

The deteriorating effect, which matrix components can exhibit on the mass spectrometric signal response when present during the ionization of the analytes, was studied in detail. The effectiveness of high turbulence liquid chromatography (HTLC) as means to minimize these matrix effects was evaluated. HTLC proved to be a highly efficient, rapid and easy-to-use method for the automated on-line sample clean-up.

To sum up, in the course of this thesis it could be demonstrated, that ESI-MS-based detection schemes are a highly valuable tool in modern bioanalytical chemistry. The methodology was demonstrated to be applicable not only to assays employing natural substrates and products, which are not amendable to standard detection schemes, but also to be capable of simultaneous multiplexed analyses. Thus, the mass spectrometric detection in enzymatic bioassays can be regarded not as a substitute for but as a very intriguing complementary method to the widely used optical or radiometric assay schemes.

Further work shall be directed to broaden the range of substrates in the multiplexed activity screening, thus providing more information about the catalytic properties of the different venom fraction and allowing a more precise classification. Additionally, the possibility of a direct coupling of a potent separation scheme with the biochemical information of the ESI-MS-based activity assay shall be investigated. Initial experiments employing a reversed-phase separation of model enzymes were not successful. The enzymes were irreversibly denatured upon contact with the stationary phase in the separation column and elution by a high organic content in the mobile phase. Therefore, an alternative separation method, which provides a full retention of the enzymes' bioactivity, possibly ion exchange chromatography or electrophoresis, has to be sought.

Summary

In the course of this work, a rapid and efficient assay scheme for the screening of relevant activities in highly complex biological samples was developed.

The general applicability of electrospray ionization mass spectrometry (ESI-MS) as means of detection in bioassays was successfully demonstrated on several enzymatic systems, such as esterases, alkaline phosphatase and a number of different proteases as well as for microperoxidase 11, which acted as a pseudo-enzyme. This clearly demonstrates the high versatility of the MS-based method, which is readily adaptable to the monitoring of different reactions. The validity of the mass spectrometric approach was tested for all enzymatic model systems by comparing reference experiments based on either fluorescence or UV/vis-absorbance detection methods. The results from the independent methods were in all cases found to be in good agreement with each other.

Besides the on-line and on-chip approaches, the at-line experimental set-up was selected for the majority of the work in this thesis. The at-line approach allows to generate complete, time-resolved reaction profiles for each enzyme-catalyzed conversion (see chapter 3). The evaluation of these profiles provides, in addition to the determination of catalytic activity, also information about the kinetic behavior of the enzymes studied. In case of

microperoxidase 11 it was found that it did not act as a true catalyst, but was inactivated after a short period of time (see chapter 4).

The most important advantage of mass spectrometric detection in comparison with the established optical or radiometric detection methods is its general applicability for multiplexed analyses. This was demonstrated in chapter 4 by the simultaneous quantitative determination of alkaline phosphatase and microperoxidase 11 in one reaction. In chapter 5, the multiplexing abilities of the MS-based detection was used to develop an assay for competitive investigations on the catalytic activity of proteases towards seven different substrates, generating unique activity patterns. On the basis of this approach, a multiplex assay for the proteolytic activity screening in snake venom was introduced in chapter 6. This “functional proteomics” methodology was successfully applied in the characterization and identification of the respective enzymatic activities in the single fractions of the pre-separated venom. This type of activity assessment is a highly promising approach towards a general high-throughput screening procedure, which allows the fast identification of lead activities in complex samples.

Furthermore, the MS-based detection was successfully employed in the development of an acetylcholinesterase-inhibition assay as screening procedure for putative inhibiting activities in the snake venom (see chapter 7). The assay scheme made use of the capability of the method to detect the consumption of the natural substrate acetylcholine and the formation of the respective product choline, which are not susceptible to the detection by any

direct optical means. Significant inhibitory activity could be assigned to two fractions of the venom. The inhibitory potential was quantified relative to that of a known acetylcholinesterase inhibitor. The findings of this study demonstrate the high value of the MS-based assay schemes in the screening for inhibitors under assay conditions, which are closely related to those found in biological relevant systems.

The inherent problem of matrix components affecting the accuracy of the ESI-MS-based detection was studied in chapter 8. The partly intolerably high deteriorating effects of matrix components on the signal response of the analytes could be effectively reduced by employing high turbulence flow chromatography (HTLC). This means of on-line sample preparation mainly known from pharmaceutical analysis could be readily adapted to the assay conditions employed in the activity screening. Limited column lifetimes as frequently reported were not found to be a major limitation, since approximately 1000 samples could be analyzed without any significant decrease in the method performance. The accuracy and sensitivity of the ESI-MS detection were significantly increased by the use of HTLC (up to a factor of 15), while the analysis time per sample remained the same, thus indicating the great potential of the method as rapid and effective sample clean-up tool.

Generally, it can be concluded, that ESI-MS is a highly valuable tool in the monitoring of enzymatic bioassays. It is very versatile, being compatible with a large variety of different enzymatic systems. Its inherent capability for multiplexed analyses makes MS-based detection schemes ideally suited for

competitive activity screening procedures, enhancing sample throughput by parallel assessment of enzymatic activities. Furthermore, ESI-MS detection allows the use of unmodified naturally occurring substrates in the assays, since it is independent from the spectroscopic or radioactivity properties of the analytes. The ruggedness of mass spectrometric detection was demonstrated by its successful application in the screening for enzyme catalytic and inhibitory activities in a highly complex biological sample. The potential problem of matrix effects for the reliable detection of analytes was demonstrated to be easily circumvented by employing HTLC as on-line means of sample preparation.

Thus, ESI-MS can probably not be expected to replace the established optical and radiometric methods of detection in enzymatic bioassays. However, mass spectrometric detection schemes offer an intriguing alternative, which complements the possibilities of existing assay schemes.

Samenvatting

In dit proefschrift wordt de ontwikkeling van een snel en efficiënt analyse-systeem beschreven voor de screening van relevante enzymatische activiteit in complexe biologische monsters.

De algemene toepassing van electrospray ionisatie massaspectrometrie (ESI-MS) als detectiemethode in bioassays is bij verscheidene enzymatische systemen toegepast, zoals esterases, alkaline phosphatase en een aantal verschillende proteases waaronder microperoxidase 11, welke als een pseudo-enzym fungeerde. Dit toont duidelijk de grote veelzijdigheid van de MS-methode aan, die direct toepasbaar is voor monitoring van verschillende reacties. De geldigheid van de massaspectrometrische aanpak werd voor alle enzymatische modellen onderzocht door vergelijking met referentie experimenten gebaseerd op fluorescentie of UV/vis detectiemethoden. De resultaten van deze methoden kwamen in alle gevallen goed overeen.

Naast de on-line and on-chip benadering werd de at-line set-up gekozen voor het belangrijkste deel van het in dit proefschrift beschreven onderzoek. De at-line benadering staat de ontwikkeling toe van complete tijd-opgeloste reactieprofielen, dit voor elke enzym-gekatalyseerde conversie (zie hoofdstuk 3). De evaluatie van deze profielen en de bepaling van de katalytische activiteit geven ook informatie over het kinetisch gedrag van de bestudeerde

enzymen. Microperoxidase daarentegen bleek zich niet als katalysator te gedragen maar werd na korte tijd inactief (zie hoofdstuk 4).

Het belangrijkste voordeel van massaspectrometrische detectie ten opzichte van de standaard optische of radiometrische detectiemethoden is geschiktheid voor ‘multiplexed‘ analyses. Dit is in hoofdstuk 4 beschreven voor de simultane kwantitatieve bepaling van alkaline phosphatase en microperoxidase 11 in één reactie. In hoofdstuk 5 is gebruik gemaakt van de multiplexed eigenschappen van de MS-detectiemethode voor de ontwikkeling van een competitieve assay van katalytische protease-activiteit tegenover 7 verschillende substraten om uiteindelijk unieke activiteitspatronen te verkrijgen. Op deze wijze wordt in hoofdstuk 6 een multiplex assay voor screening van proteolytische activiteit in slangengif geintroduceerd. Vervolgens kon deze methodologie succesvol worden toegepast bij de karakterisering en identificatie van de enzymatische activiteit in de afzonderlijke fracties van het gif. Dit type beoordeling van activiteit kan leiden tot een ‘high throughput’ screening methode welke de snelle identificatie van belangrijke enzymatische activiteit in complexe monsters mogelijk maakt.

Bovendien is de MS-gebaseerde detectiemethode gebruikt in de ontwikkeling van een assay voor acetylcholinesterase inhibitor als screening methode voor potentiële activiteit in slangengif. (zie hoofdstuk 7). Dit assay maakt gebruik van de mogelijkheid om detectie van substraatconsumptie (acetylcholine) en productvorming (choline) mogelijk te maken welke ongeschikt zijn voor directe optische detectie. In het gif konden 2 fracties worden aangewezen met

significante remmingsactiviteit. Deze eigenschap kon worden gekwantificeerd naast de activiteit van een bekende acetylcholinesterase-remmer. De resultaten van deze studie tonen duidelijk de waarde van de MS-methode in de screening van remmers aan onder vergelijkbare condities die in biologisch relevante systemen gevonden wordt.

Het probleem van matrix componenten die de nauwkeurigheid van de ESI-MS methode beïnvloeden wordt in hoofdstuk 8 behandeld. De storingsinvloeden van matrix componenten kon voor een groot deel door middel van high turbulence flow chromatography (HTLC) worden gereduceerd. Deze on-line monstervoorbewerkingsmethode, voornamelijk bekend in de klinische chemie, kon direct worden aangewend in de screeningsmethode. De beperkte levensduur van de kolommen vormden geen belangrijke beperking. Ongeveer 2000 monsters werden probleemloos geanalyseerd zonder significant prestatieverlies. De nauwkeurigheid en gevoeligheid van de ESI-MS methode kon worden verbeterd door toepassing van HTLC bij gelijkblijvende analysetijd per monster. Dit toont het grote potentieel van de methode aan als snelle, effectieve clean-up tool.

Algemeen kan worden geconcludeerd dat ESI-MS een waardevolle tool is bij de monitoring van enzymatische bioassays. Het is een erg veelzijdige methode, compatibel met een grote variëteit aan verschillende enzymatische systemen. De toepasbaarheid voor multiplexed analyses maakt de MS-methode geschikt voor competitieve screenings met grotere monsterdoorvoersnelheid door parallele beoordeling van enzymatische

activiteit. Bovendien kan men in de assays gebruik maken van ongemodificeerde natuurlijke substraten vanwege de onafhankelijkheid van spectroscopische of radioactieve eigenschappen van de analieten. De robustheid van de massa-spectrometrische detectie werd aangetoond door haar succesvolle toepassing in de screening van enzymkatalytische en remmings-activiteit in een complex biologisch monster. Het probleem van matrixeffecten voor een betrouwbare detectie kon eenvoudig worden ondervangen door gebruik van HTLC als een on-line monstervoorbewerkings-methode.

ESI-MS kan waarschijnlijk geen optische en radiometrische detectiemethoden in enzymatische bioassays vervangen. Niettemin kan massaspectrometrische detectie een interessante aanvulling voor bestaande assays vormen.

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List of Publications

Original Papers

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