3. Methods

3.1 General part

3.1.1 Matrix-assisted-laser-desorption-ionization-time- of- flight-mass-spectrometry (MALDI-TOF-mass spectrometry)

MALDI-TOF mass spectrometry is used to determine the molecular weight of molecules such as peptides and proteins. This method determines the mass-to-charge ratio (m/z) of ions. Sample molecules are ionized in the source and accelerated by an electric field into the analyzer. In time-of-flight (TOF) mass spectrometry (figure 1a) the analyzer is a chamber under vacuum that contains no electric fields. The ions drift through the analyzer with the kinetic energy obtained from the potential energy of the electric field. They traverse the analyzer in a time which depends upon their m/z ratios. The detector, positioned at the end of the analyzer, measures the arrival time of the ions. All ions obtain the same kinetic energy (K.E.) from the potential energy of the electric field. The equation $m/z=2*K.E./v^2$ describes the relation between molecular mass (m) and velocity (v).Therefore ions of lesser m/z have a higher velocity and arrive first, followed by ions of greater m/z and lower velocity (figure 1b).

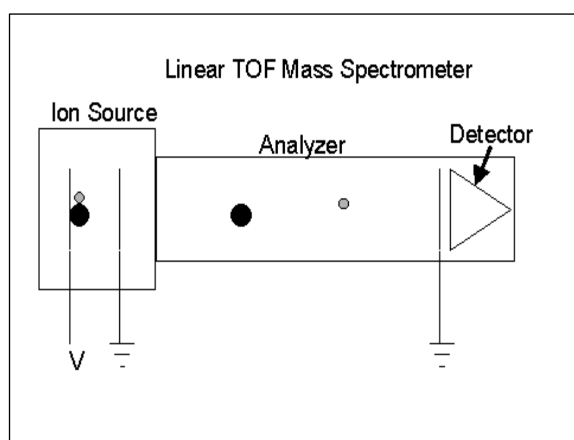

Figure 1a Scheme of the general components of a mass spectrometer

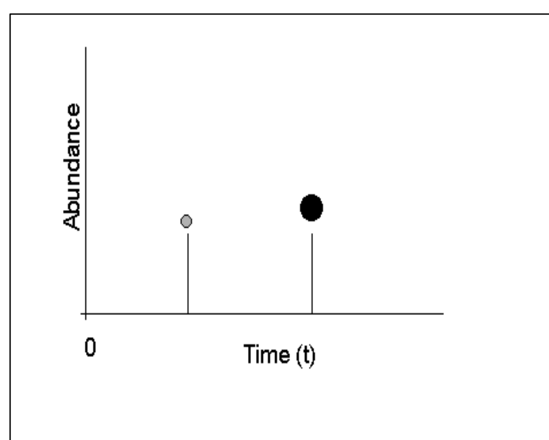

Figure 1b Plot of abundance versus time for ions detected in TOF mass spectrometer

Ions that have low molecular masses have a lower time-of-flight than ions with high molecular masses. By comparing the time-of-flight of know reference masses with the time-of-flight of the sample ions the molecular mass of the sample ions can be calculated. MALDI-TOF mass spectrometry allows the measurement of molecular masses of compounds with high accuracy and sensitivity.

3.1.2 MALDI-TOF- mass-spectrometry-assisted enzyme screening (MES) system

The MES-system is an assay system which can be used to detect enzymatic activities (41). The analytical procedure consists of five steps. First, proteins are covalently immobilized to cyanogens bromide activated sepharose beads via their amino groups (figure 2, step *1*). After immobilization of the proteins the enzymatic activities are detected by incubating the beads with the reaction specific enzyme substrates (figure 2, step *2*). If the target enzyme is present in the immobilized protein fraction its specific substrate will be converted into its expected reaction products. This reaction starts as soon as the immobilized protein fractions are mixed with their specific substrates (figure 2, step *3*). After defined incubation times aliquots are taken from the reaction mixture (figure 2, step *4*) and analyzed with MALDI-TOF mass spectrometry (figure 2, step *5*). The target enzyme is present if the mass signal from the expected reaction product can be detected in the MALDI-TOF mass spectra. The quantification of enzyme activities is performed by calculating the ratio of the signal intensity of the product signal and the signal intensity of the substrate signal.

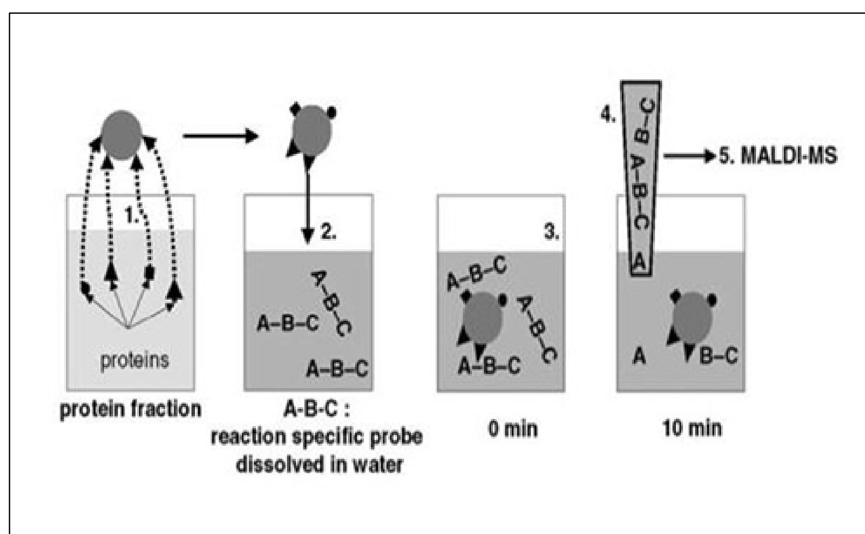

Figure 2 Scheme of the MES procedure

$$relative\ intensity = \frac{signal\ intensity\ of\ \ product\ signal}{signal\ intensity\ of\ \ substrate\ signal}$$

During the time- dependent incubation of an immobilized enzyme the substrate is converted to a reaction product. Therefore in the mass spectra of the reaction mixtures the signal intensity of the product signal increases while the signal intensity of the substrate decreases. Accompanying with the change of the product / substrate signal intensity, the calculated relative intensity also changes. The term *maximum of enzyme activity* refers to the maximum of the relative intensity and therefore to the maximum of ratio of the product signal intensity/ substrate signal intensity.

This quantification method is semi-quantitative, nevertheless precise enough to detect significant differences in the level of enzyme activities.

3.1.3 Chromatography

Liquid chromatography is a purification method available for separation of proteins. Chromatography is a separation method in which the different components in a sample migrate through a column at different rates. There are basically two different kinds of mechanism for chromatography: adsorption, in which the sample molecules are adsorbed onto the chromatographic medium through noncovalent binding (such as ion exchange, hydrophobic interaction), and nonadsorption (such as size exclusion chromatography).
Adsorption chromatography is based on the distribution of a sample molecule between a stationary (synonymous also: solid) and a liquid phase. The molecules distribute between the stationary and the liquid phase according to their affinity for the matrix: molecules with greater affinity for the stationary phase will have higher concentration in the stationary phase and lower concentration in the liquid phase. The term “affinity” refers to the attraction between the sample molecule and the stationary phase. It depends on the physical and chemical properties of the sample molecule and the stationary phase and the resulting interactions between them. Because different molecules have different distribution characteristics (as a result of different physical and chemical properties), it is possible to separate the molecules from each other.

3.1.3.1 Sample displacement chromatography

In chromatographic purification steps based on adsorption principles a sample displacement mode can be used in order to achieve a fast purification of the main contaminants from the target protein (42). Briefly sample displacement utilizes the competition of sample molecules for their binding onto the functional groups of the stationary phase of a chromatography media. A distinct amount of chromatography media is aliquoted into same size portions. Then an amount of sample molecules that exceeds the binding capacity of the stationary phase of the chromatography media is applied to the first aliquot of the chromatography media. Sample molecules that have a highest affinity to the functional groups will bind first. The supernatant of the first chromatography media aliquot contains sample molecules with lesser affinity that will bind to the next media aliquots. This supernatant is applied to the next media aliquot and the containing sample molecules will start to compete for their binding onto the functional groups of

the media again. Now molecules with lesser affinity have a chance to bind. The resulting supernatant is applied to the next media aliquots and so on. As a result the sample molecules are not just separated by their ability to bind onto a stationary phase but also according to the strength of their affinity to the stationary phase during the course of sample displacement chromatography

3.1.3.2 The protein- purification- parameter-screening system (PPS)

The protein-purification-parameter screening system is an automated platform technology employed for the determination of optimal parameters for the chromatographic purification of a target protein (43). The purification strategy of a protein should involve as few steps as possible in order to receive a high specific yield with high recovery of the target protein. The resolution capacity of a chromatographic purification steps depend on a number of individual parameters such as pH and ionic strength of the liquid phase of the chromatographic purification step. Also the properties of the target protein such as pI or distribution of hydrophobic/ and hydrophilic amino acid residues play an important role in the search for optimal protein purification parameters. Systematic variation of all the relevant parameters will help to find the appropriate purification parameters for the purification of a protein. The protein-purification-parameter-screening system is based on a batch chromatography that is performed in deep-well plates. Several stationary phases of a chromatography media are aliquoted into a deep-well plate. Then the stationary phases are equilibrated with individual buffers varying in pH, ionic strength or composition of additives. After equilibration of the chromatography media the protein sample is applied to several stationary phases. Non binding-molecules are removed by washing the stationary phase with their individual equilibration buffers. The absorbed proteins are eluted with an appropriate elution buffer in order to achieve to most effective purification of an individual protein. During the course of purification of a target protein from crude extract to a homogeneous protein fraction the PPS experiments are done as a set of batch chromatography prior to the actual preparative chromatographic purification step. Various chromatography parameters are tested during PPS in order to get a guideline which can be used for the upcoming preparative chromatographic purification step of the target molecule.

3.1.3.3 Ion exchange chromatography

Types of adsorption chromatography are classified by their principles of interaction. Ion exchange chromatography is based on electrostatic interaction between the charged molecules in the sample and the charged ion exchange matrix. Proteins are made up of amino acids that contain various chemical groups attached to a peptide backbone. The charge of these groups is dependent upon the pH of the solvent in the mobile phase. The pH value at which they have zero net charge is termed "isoelectric point". Below this point the net charge of a protein is positive and the protein will bind to a cation exchanger, whereas above this point the net charge of a protein is negative and the protein will bind to an anion exchanger. The ion exchange resin (solid support matrix) contains electrically charged ligands, such as quaternary ammonium (anion exchanger) or sulfonyl groups (cation exchanger). Associated with each linked charge is a counter ion of opposite charge, bound by ionic attraction.
Figure 3 illustrates the mechanism of ion exchange. Charged regions of the protein carrying a charge of opposite polarity to the resin will be attracted to the stationary phase, displace the counter ion and become bound to the resin by ionic interaction. Noncharged proteins will not become attached to the resin and may be separated by washing the resin with mobile phase. The protein bound by ionic interaction can be removed from the resin by displacement with an ion in the mobile phase having a stronger affinity for the stationary phase than the protein does. This displacement is termed elution and can be achieved by washing the stationary phase with high salt solvents. The displacement occurs in the order of relative affinity. The ion with the greater affinity for the resin displaces the one with a lesser affinity.

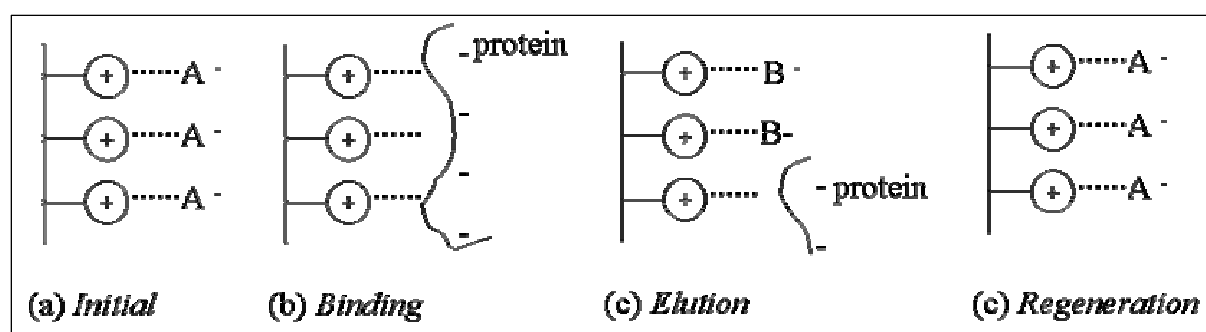

Figure 3 Scheme illustrating the mechanism of ion exchange. (*a*) Anion exchange resin with negatively charged counter ions (*b*) Negatively charged protein associated with resin (*c*) Elution of protein from resin. (*d*) Regeneration of resin to original counter ion

3.4.3.4 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography is a kind of adsorption chromatography that is based upon interactions between hydrophobic patches on the surface of proteins and the hydrophobic ligands (alkyl or aryl groups) covalently attached to the HIC gel matrix chromatography media. In the presence of moderately high concentrations of anti-chaotropic salts in the buffer protein and salt molecules compete for the water molecules in order to keep their hydrate shield. If the protein molecules loose their hydrate shield they start to expose their hydrophobic patches to their surrounding liquid micro environment. This exposure promotes the binding of the hydrophobic patches to the hydrophobic ligands of the HIC gel matrix chromatography media. Hofstee proposed the term ''hydrophobic chromatography'' with the implicit assumption that the mode of interaction between proteins and the immobilized hydrophobic ligands is similar to the self association of small aliphatic organic molecules in water (44)). Elution of the adsorbed proteins is achieved by washing the HIC gel media with low/ non salt containing buffers. Protein molecules then gain back the water molecules for their hydrate shield and with this their solubility in an aqueous environment.

3.1.3.5 Hydroxyapatite chromatography

Hydroxyapatite chromatography is a kind of chromatography that also uses adsorption mechanism for the separation of biomolecules. Hydroxyapatite $(Ca_5(PO_4)_3OH)_2$ is a form of calcium phosphate that can be used for the separation and purification of proteins. It carries positive (Ca^{2+}) and negative (PO_4^{3-}) charges where proteins are able to bind. The separation mechanism is not exactly understood yet but it seems that acetic regions of a proteins bind preferably to the calcium ions, whereas basic regions of a protein bind to the phosphate ions. The binding of a protein does not only depend on its whole charge but also on the charge distribution on the protein surface. The bound proteins can be eluted by washing the resin with high phosphate concentration in the elution buffer.

3.1.3.6 Affinity chromatography of glycoproteins

Affinity chromatography is based on specific interaction between the sample molecule and a complementary structure bonded to the stationary phase. In eukaryotic cells most proteins are

subject to post-translational modification, of which glycosylation is one of the most common forms. Purification of proteins selectively utilizing their glycan components as a capture component is commonly done using affinity chromatography. Glycoproteins bind reversible, via specific sugar residues, to a group of proteins known as lectins. Lectins are proteins of plant origin, often from pulses, which have a high affinity to the sugar chains of glycoproteins. The binding interaction between a lectin and a specific sugar residue is analogous to that between an antibody and an antigen. Chromatography resins with immobilized resin are commercially available. Glycoproteins bound to lectin resin may be eluted by washing the resin with a competitive binding substance or a high salt solvent.

3.1.3.7 Inhibitor affinity chromatography of enzymes

Proteins that have enzymatic activities can be purified by using inhibitor affinity chromatography. An enzyme inhibitor is a molecule that is able to bind specifically to the recognition site of an enzyme and as a result inhibits the enzyme activity. The reversible binding of an inhibitor to an enzyme can be used for purification of that enzyme. At first an enzyme specific inhibitor is covalently coupled via a cross-link reaction to a resin. After conditioning of the resin the protein sample is applied. The enzymes that have the specific recognition site for the inhibitor bind to the immobilized inhibitor. Proteins that do not have the specific recognition site will not bind and are washed out. The elution of the bound enzymes is achieved by washing the resin with the free inhibitor, which competes with the immobilized inhibitor for the binding sites of the enzymes. As a result the enzymes are removed from the resin. Another elution mode can be carried out by washing the resin with a low pH buffer. Low pH changes the conformation of the enzyme and thus loosens the interaction between the enzyme and the inhibitor.

3.1.3.8 Size exclusion chromatography

The non-absorbent size exclusion chromatography separates molecules according to differences in size as they pass through a gel filtration media packed in a column. The separation is based on the different rates of diffusion of sample molecules of different sizes in the porous matrix of the gel filtration media. Those molecules which are small enough to diffuse an appreciable distance into the matrix will be retarded and elute later than larger molecules. Sufficiently larger molecules have a lower ability to diffuse into the pores of the matrix and will be less retarded and elute before smaller molecules.

High resolution size exclusion chromatography can be used for the determination of molecular weight of biomolecules. Results from a gel filtration are usually expressed as an elution profile or chromatogram that shows the variation in concentration (typically in terms of UV absorbance at A_{280} nm) of sample components as they elute from the column in order of their molecular size. The partition coefficient K_{av} that can be calculated is related to the size of a molecule. Over a considerable range there is a linear relationship between K_{av} and $\log_{10} M_r$ (molecular weight) of molecules. By plotting the K_{av} against the decimal logarithm of the molecular weight of a set of standard proteins a calibration curve can be prepared. The molecular weight of an unknown substance can be determined from the calibration curve once the K_{av} of the unknown substance is calculated. The K_{av} can be calculated as follows:

$Kav= (Ve-Vo)/ (Vt-Vo)$.

Ve is the elution volume of the protein, *Vo* is the column void volume (equal to the volume of the mobile phase), *Vt* is the total bed volume (equal to the volume of the solid phase). Figure 4 illustrates the relation between K_{av} and molecular weight.

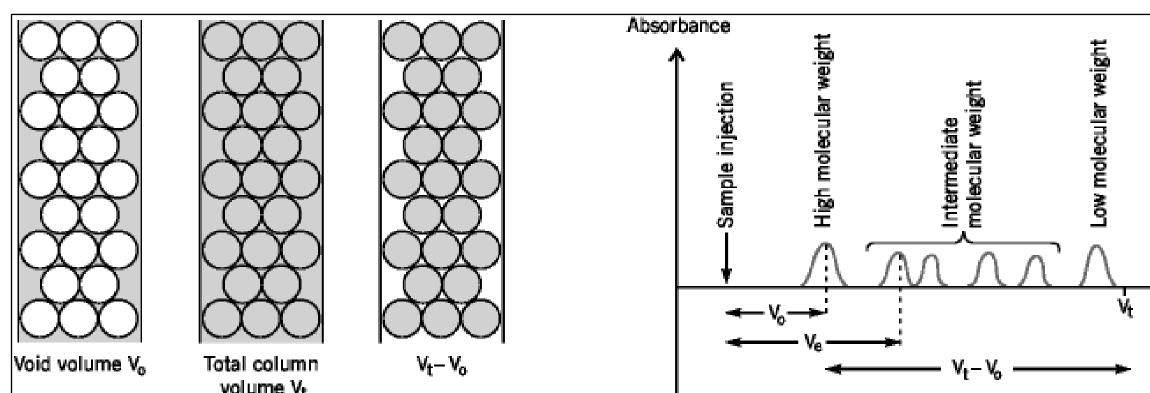

Figure 4 Scheme of the approximate relationship between *Vo, Vt*, *Ve* and molecular weight of sample proteins

3.1.4 One- dimensional gel electrophoresis

One-dimensional electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method with which to identify and monitor proteins during purification and to assess the homogeneity of purified protein fractions. SDS-PAGE is routinely used for the estimation of protein subunit molecular weights and for the determining the subunit composition of purified proteins (45).

Proteins are treated with the anionic detergent sodium dodecyl sulphate (SDS). The detergent binds tightly in constant ration mg SDS/mg protein to most proteins. The SDS imparts a negative charge to the resulting complex (46). Treatment with SDS and concomitant treatment with

disulfide-reducing agents, such as β-mercaptoethanol denatures proteins and breaks them down to their constituent subunits. In an electrophoretic separation the negative charged SDS-polypeptides are caused to migrate to the anode of the electrophoresis chamber under influence of an externally applied electric field. The surrounding gel matrix acts as a molecular sieve and causes the retardation of the moving SDS-polypeptides. The opposing interaction of the electrical force and molecular sieving leads to differential migration rates of the SDS-polypeptides. The migration rate of the SDS-polypeptides is inversely proportional to the logarithm of their molecular weight (47). Low- molecular-weight SDS-polypeptides migrate faster than larger one. The molecular weight of a protein subunit can be estimated from its relative mobility in a calibrated SDS-PAGE gel.

3.1.5 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis separates proteins in two dimensions- in the first dimension according to their isoelectric points using isoelectric focussing (IEF), followed by separation in the second dimension according to their molecular weights using the previously described SDS-PAGE.

Proteins are amphoteric molecules that carry positive, negative or zero net charges depending on the pH of their local environment. The overall charge of a protein is determined by the ionizable acidic and basic chains of its constituent amino acids and prosthetic groups. The net charge on a protein is the sum of all its positive and negative charges. There is a specific pH for every protein at which the net charge it carries is zero. This isoelectric pH value is termed *isoelectric point* (p*I*) and is a characteristic physiochemical property of every protein (48). Proteins are positively charged in solution at pH below their p*I* –value and negatively charged above their p*I* –value. Two-dimensional gel electrophoresis uses an immobilized pH-gradient during isoelectric focussing. In a pH-gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with positive net charge will migrate towards the cathode until it reaches its p*I*. A protein with negative net charge will migrate toward the anode until it reaches its p*I*. If a protein should diffuse away from its p*I*, it immediately gains back charge and moves back. This focusing effect of IEF, which concentrates proteins at their p*I*s allows the proteins to be separated on the bases of small charge differences. After IEF, SDS-Page as the second dimension of separation can be performed.

3.1.6 Identification of proteins by MALDI-TOF peptide mass fingerprint

Proteins which were separated to homogeneity by one or two dimensional electrophoresis can be identified using mass spectrometric techniques such as MALDI-TOF. The principle of peptide mass fingerprint is- if two proteins have identical primary sequences, then the cleavage of each protein with a site-specific endopeptidase will yield identical peptide fragments with identical molecular masses (49). The general procedure of peptide mass fingerprint is outlined in figure 5.

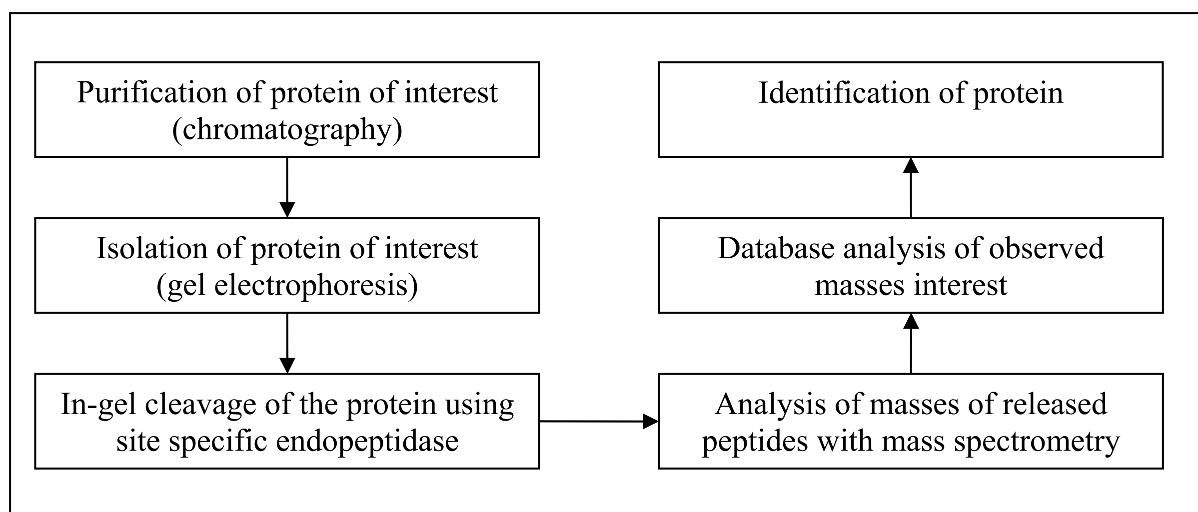

Figure 5 Scheme of steps necessary for identification of proteins using peptide mass fingerprint

Peptide mass fingerprint is a method to identify proteins, from which the sequences are already present within a sequence database. The peptides are experimentally generated applying site specific cleavage reagents (either enzymatic or chemical) to the protein of interest. The experimentally generated peptide masses are matched with theoretical peptide masses calculated from each sequence entry in the database if the database sequences had been cleaved with the same specificity as the reagent in the experiment. A score is calculated to provide a measure of the fit between the observed and expected peptide masses so that the unknown protein can be matched and identified. In order to identify a protein with peptide fingerprinting the highest mass accuracy should be employed. The specificity of the cleavage is also critical. Furthermore post-translational or artifactual modification of the protein of interest should be taken in consideration since they alter the molecular masses of the resulting proteolytic peptides.

3.1.7 Identification of proteins by nano Liquid-Chromatography (nanoLC) coupled with Electrospray-Ionization mass spectrometry (ESI-MS/MS)

Fragmentation of gas- phase peptide ions often provides extensive sequence specific information. The peptide mixture derived from enzymatic in-gel-digestion is applied to a nanoLC-Reversed-Phase column (RP). This chromatography separates the proteolytic peptides according to their hydrophobic interaction with the alkanylated porous silica matrix of the column. The eluate of the column is directly applied to the Electrospray- ionization (ESI) source of the mass spectrometer. ESI produces ions through the application of high voltage to a stream of liquid emitted from the opening of the capillary coming from the RP-column. A cone of highly charged droplets is sprayed from the opening and is sampled by an ion trap (IT) mass spectrometer. The IT mass spectrometer is capable of performing collision induced dissociation (CID). Selected single ions are trapped, ionized and made to collide with neutral gas molecules of argon or helium. The precursor ions fragment into product ions. Each peptide will generate a sequence- specific MS/MS spectrum. The MS/MS spectrum is a plot of the frequency of the peptide fragmentation, where the m/z value of the fragments represent specific amino acid sequence fragment occurrences. The original peptide mass and the fragmentation pattern are needed for the protein identification by correlation with a database entry. Algorithms such as Mascot, SEQUEST or BLAST are used for the automated approach to search databases with MS/MS spectra data.

3.2 Purification of Angiotensin-II-generating enzymes

Figure 6-9 show an overview of the experimental steps that have been carried out within the scope of this work. Unless otherwise stated all reagents were purchased from Sigma. For the detection of angiotensin-II-generating activity the MES-assay was used. Figure 6 shows the steps necessary to establish the MALDI-TOF- mass-spectrometry-assisted enzyme screening (MES) assay in order to detect angiotensin-II-generating enzyme activities (Part I). The capability of the MES-assay to detect angiotensin-II-generating was demonstrated using a pure enzyme (*point 2* angiotensin- converting enzyme) and a crude protein extract (*point 1* porcine renal tissue homogenate). A calibration curve was prepared (*point 4*) for quantification of the angiotensin-II-generating utilizing the MES-assay. Therefore the amount of angiotensin-II in a reaction solution was plotted versus their corresponding angiotensin-II signal intensities. The detection limits of enzyme activities using the MES-assay were compared with the detection limits of a conventional fluorometric assay system using angiotensin converting enzyme as a model enzyme (*point 5*). The action of specific enzyme inhibitors on the angiotensin-II generating activity of porcine renal tissue was investigated using the MES-assay (*point 6*).

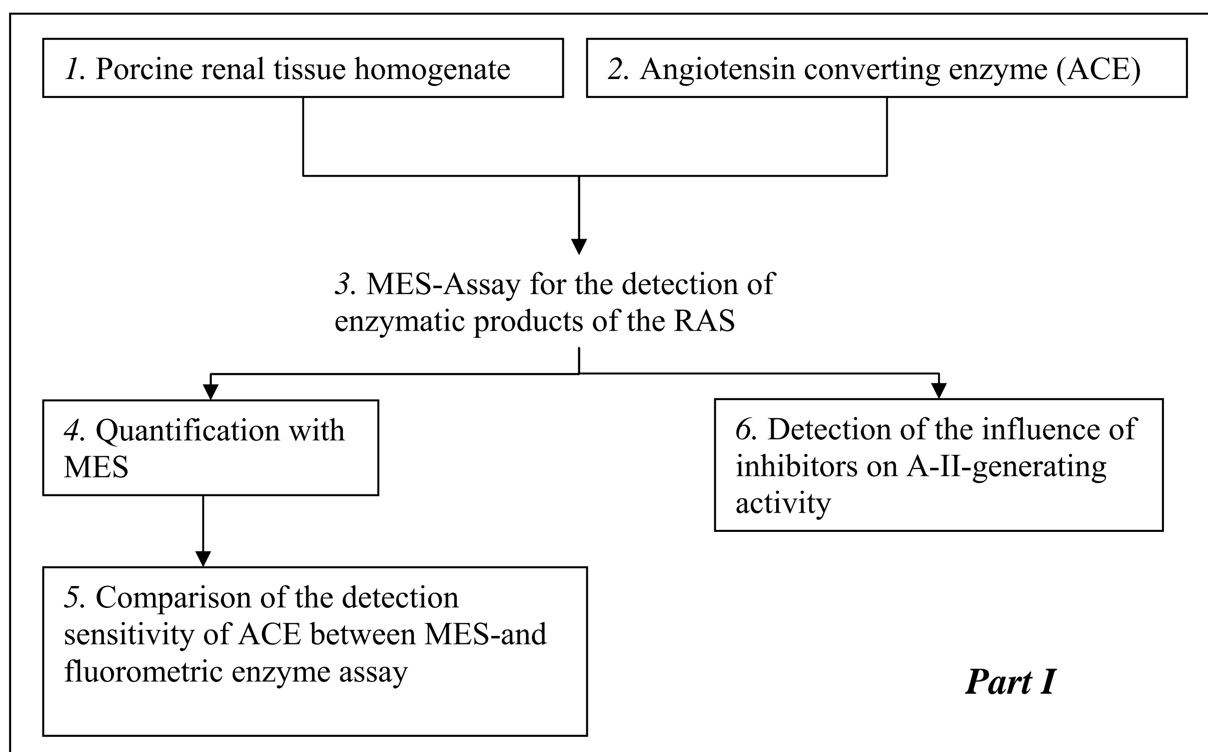

Figure 6 Experiments used for the establishment of MES-assay-system

Figure 7 shows the first purification path and the purification steps that were used in order to purify two independent angiotensin-II-generating fractions from porcine renal tissue (Part II).

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Porcine kidneys *(point 1)* were purchased from the local slaughterhouse and lyophilized. The porcine renal tissue lyophilizate was homogenized *(point 2)* and centrifuged. The supernatant was processed through a set of chromatographic steps consisting of anion exchange chromatography *(point 3)*, hydroxyapatite chromatography *(point 4)* and lectin affinity chromatography *(point 5)* and yielded two independent angiotensin-II-generating protein fractions. Protein purification scouting experiments (PPS) were performed preliminary to each chromatographic step in order to find optimal chromatographic purification parameters. Fraction I was further purified using chymostatin-antipain affinity chromatography *(point 6)* and the resulting angiotensin-II-generating protein fraction was applied to SDS-PAGE *(point 7)*. Fraction II was further purified using size exclusion chromatography *(point 8)* and the resulting angiotensin-II-generating protein fraction was applied to two-dimensional electrophoresis *(point 9)*.

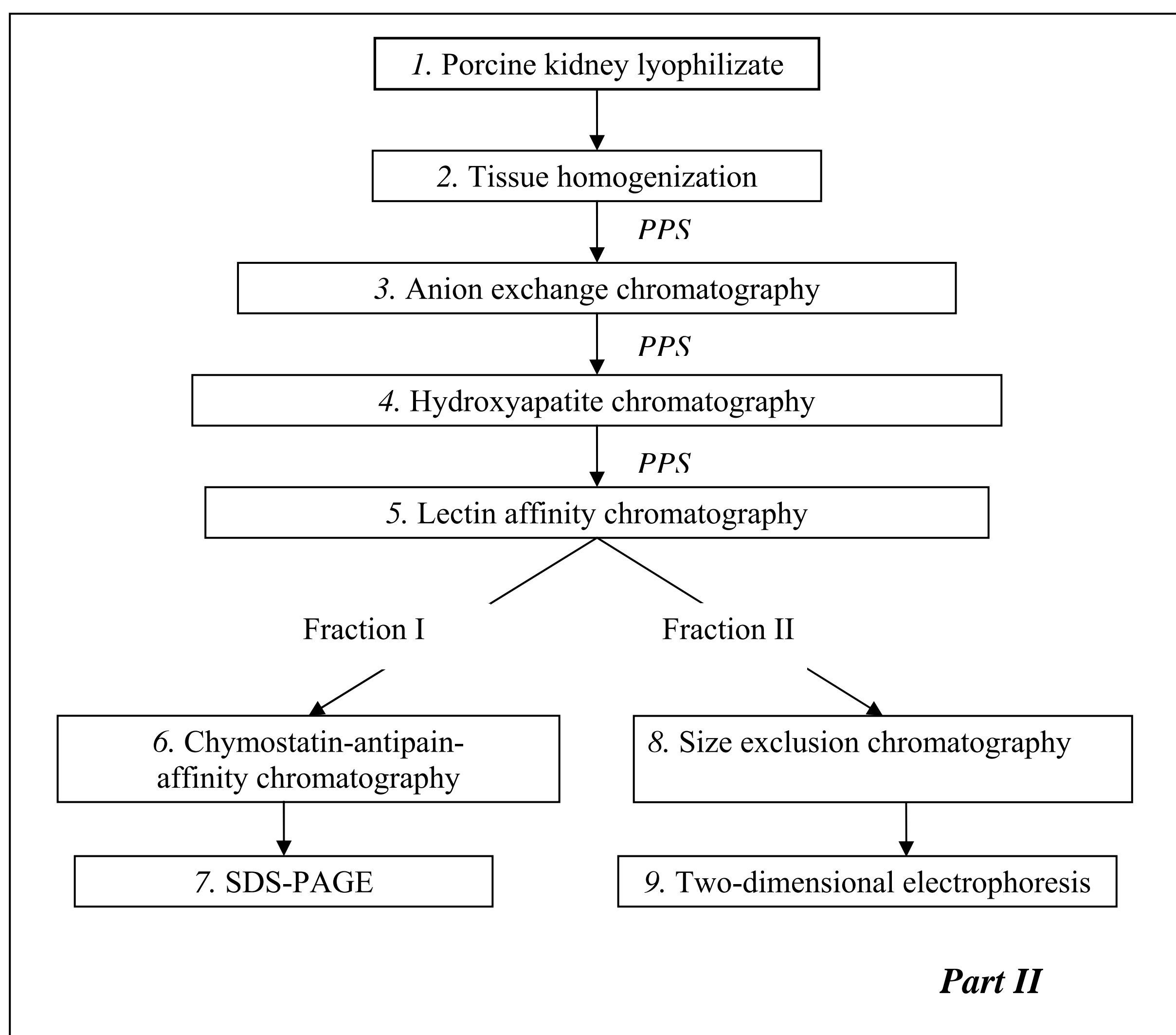

Figure 7 Purification schemes of the two fractions (Fraction I and II) with angiotensin-II-generating activity

Figure 8 shows the second purification path and the purification steps that were used for the purification of a further angiotensin-II-generating fraction from porcine renal tissue (Part III). Porcine kidneys *(point 1)* were purchased from the local slaughterhouse and lyophilized. The porcine renal tissue lyophilizate was homogenized *(point 2)* and centrifuged. The supernatant was processed through a set of chromatographic steps consisting of anion exchange chromatography *(point 3)*, lectin affinity chromatography *(point 4)* and aprotinin affinity chromatography *(point 5)*. As a result an angiotensin-II-generating Fraction III was yielded. One aliquot of this fraction was applied to SDS-PAGE *(point 6)*. A second aliquot was used for a test on substrate specifity *(point 7)*. A third aliquot was applied to size exclusion chromatography *(point 8)* in order to determine the molecular weight of the enzyme responsible for the angiotensin-II-generating activity of Fraction III.

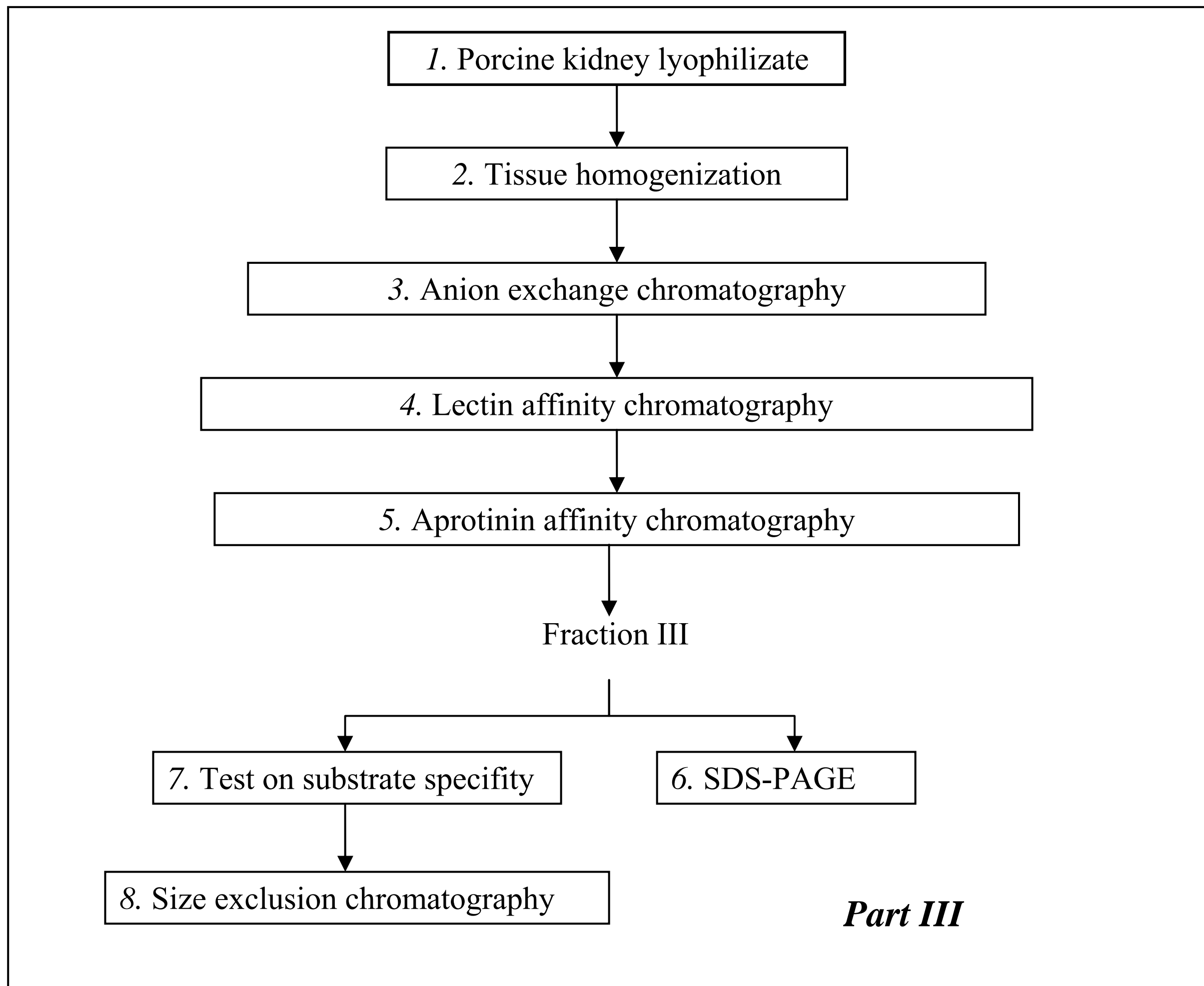

Figure 8 Purification scheme of the angiotensin-II-generating Fraction III

Figure 9 shows the experiments that led to the protein identification of the enzymes responsible for the angiotensin-II-generating activity in Fraction I, II and III (PART IV). The respective protein bands from the SDS-PAGE of Fraction I (point 1) and fraction III (point 3) and the respective protein band from the two-dimensional electrophoresis of Fraction II (point 2) were excised and applied to in-gel-digestion with specific endopeptidase *(point 4).* The proteolytic peptides of Fraction I, II, and III were applied to peptide mass fingerprint experiments *(point 5).* Furthermore proteolytic peptides of Fraction I and Fraction III were applied to nanoLC-ESI-MS/MS *(point 6)* in order to determine sequence information. The determined information from peptide mass fingerprint and nanoLC-ESI-MS/MS was used for protein database analysis *(point 7).*

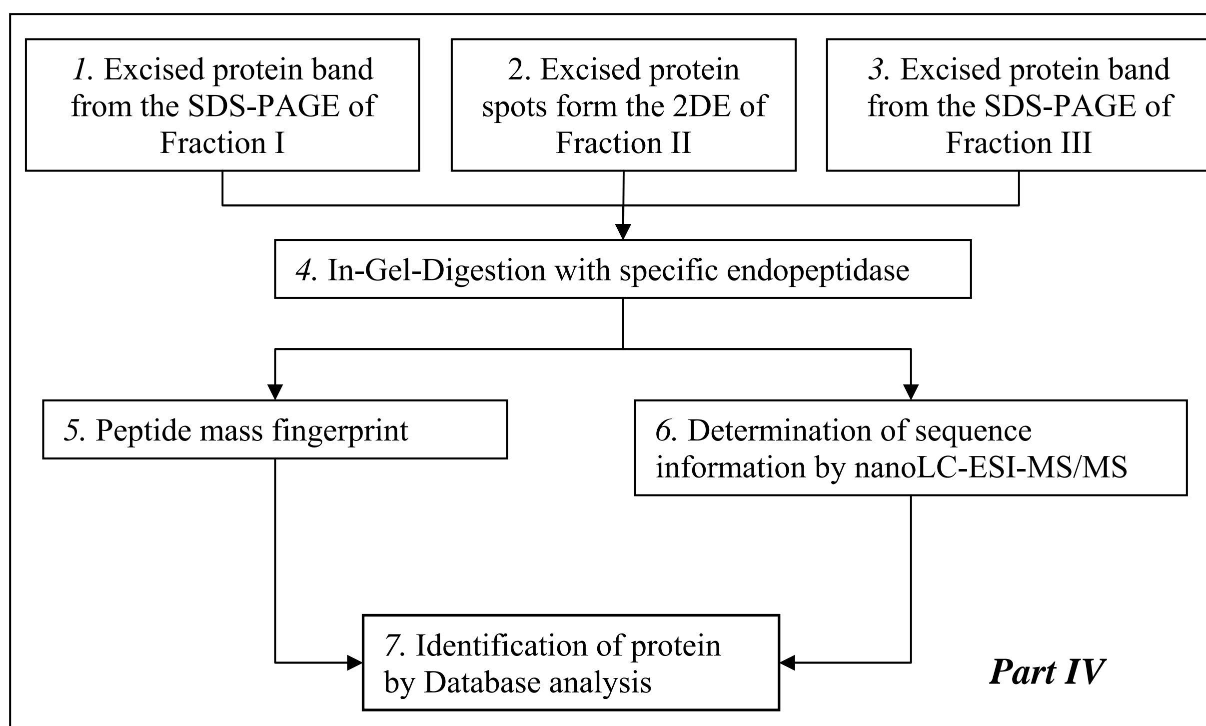

Figure 9 Protein identification experiments, 2DE: two-dimensional electrophoresis

3.2.1 Part I- Detection of enzymatic products of the renin-angiotensin-system (RAS) with the MES- Assay

3.2.1.1 Tissue homogenization and preparation of the protein extract

Blood free porcine kidneys were placed in ice-cooled physiological saline solution immediately after excision. The kidneys were cut into small pieces (1 cm^3), frozen in liquid nitrogen and stored at -80 °C for 24 h. The frozen tissue was lyophilized and powdered. The freeze-dried powder was homogenized for 2 min at 4°C with an ultra thorax in a 10fold volume of 50 mM AMPSO (pH 9). The homogenate was centrifuged at 30000 g for 1 h at 4°C. The pellet was discarded. The protein concentration of the supernatant was determined using the Bradford method.

3.2.1.2 Detection of angiotensin-II-generating enzyme activity

10 mUnits of porcine ACE (Sigma) in 25 µL HPLC-grade water and 50 µL of porcine renal tissue extract (protein concentration 20 µg/µL) was each mixed with 150 µL immobilization buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) and 50 µL CNBr-activated sepharose beads

(Amersham Bioscience). The mixtures were incubated for 2h at room temperature on an overhead rotor. After immobilization the free binding sites on the sepharose beads were blocked with 150 µL blocking buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, 0.2 M glycine pH 8.3) for 2 h at room temperature. After blocking the beads were washed four times with HPLC-grade water. A control (glycine immobilized beads) was processed the same manner.

20 µL of the sepharose beads containing the immobilized ACE, porcine renal tissue protein extract as well as the control beads were transferred each into individual wells of a 96-well microtiter plate. 30 µL of 10^{-5} M solution of the reaction specific substrate angiotensin-I (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}) (Bachem) was added to each sample. The wells containing the reaction mixtures were sealed and incubated at 37 ° C in a shaker. For the mass spectrometric analysis 1 µL aliquots were taken from the reaction mixture after 0, 30, 60, 120 and 240 minutes.

For the MALDI-TOF MS sample preparation of the reaction mixture the 1 µL aliquots of the reaction mixture were applied onto a 384- MPT AnchorChip target (Bruker). After evaporation of the reaction mixtures one µL of matrix solution (20 mg/ mL α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile, 0.1% trifluoroacetic acid in water) was transferred to the dry sample. After drying the AnchorChip target was introduced into a Bruker Reflex III reflectron type time-of-flight mass spectrometer. Positively charged ions were analyzed using the reflector mode and delayed ion extraction. The spectra were recorded with a 2-GHz sampling rate, 150 ns extraction delay time and deflection was used to suppress ions smaller than 800 m/z and bigger than 1400 m/z. A nitrogen laser with an emission wavelength of 337 nm and 3 ns pulse was used. For each sample, 100 single-shot spectra were accumulated, which result from 5 different spots per sample (20 spectra per spot). The further data processing and analysis was performed automatically using the Reflex III software package XMASS 5.1. Automated peak picking was performed using the SNAP algorithm in order to label the first monoisotopic peak of the peptide mass signal. The ration of the signal intensity of the proteolysis product angiotensin-II was calculated with:

$$relative\ intensity\ A\ II = \frac{signal\ intensity\ of\ \ product\ signal\ AII}{signal\ intensity\ of\ \ substrate\ signal\ AI}\ .$$

The highest ration of the signal intensity of angiotensin-II was named *maximal angiotensin-II-generating activity* and used as a criterion for the comparison of the angiotensin-II enzyme activities during the course of protein purification.

3.2.1.3 Correlation between enzyme activity and MES-signal intensity

In order to distinguish enzyme fractions with high and low angiotensin-II-generating activity the correlation between enzyme activity and MES-signal was investigated. Therefore 10 mUnits of porcine ACE (Sigma) was immobilized on 50 µL CNBr-activated sepharose beads (Amersham Bioscience) as described in 3.2.1.2. A control (glycine immobilized beads) was processed the same manner. The sepharose beads containing the immobilized ACE were split into two 25 µL aliquots. The aliquots of the immobilized ACE as well as the control were transferred each into individual wells of a 96-well microtiter plate. 30 µL of 10^{-5} M solution of the reaction specific substrate angiotensin-I (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}) (Bachem) was added to each sample. To one aliquot of the immobilized ACE 10 µM (final concentration) angiotensin converting enzyme specific inactivator Cyano-Phe-Phe-OH (Bachem) was added. The wells containing the reaction mixtures were sealed and incubated at 37 ° C in a shaker. For the mass spectrometric analysis 1 µL aliquots were taken from the reaction mixture after 1, 2, 4, and 24 hours. The MES-assay was carried out as described in 3.2.1.2. The relative intensities of the angiotensin-II signal during the time course dependent incubation of the reaction mixtures was calculated as described in 3.2.1.2. The angiotensin-II generating activities (determined by the MES-assay) of the immobilized porcine ACE in presence and absence of the specific inactivator were plotted versus the incubation times.

3.2.1.4 Comparison of the detection sensitivity of ACE between MES- and fluorometric enzyme assay

For the fluorometric assay of porcine ACE a 1:10 dilution series of the enzyme in water was prepared with the following concentrations 20 ng/µL, 2 ng/µL, 0.2 ng/µL, 20 pg/µL and 2 pg/µL.

Following other solutions were made: substrate solution (50 mg/mL Hippuryl-His-Leu-OH (Bachem) in 1 mL methanol), assay buffer (0.4 M sodium borate, 0.9 M sodium chloride pH 8.3), working solution (40 µL substrate solution in 9.96 mL assay buffer), derivatization buffer (0.1 M sodium tetra borate pH 9.5), OPA-solution (27 mg ortho-phtaldialdehyde first dissolved in 500 µL ethanol than add 4.5 mL derivatization buffer and 50 µL β-mercaptoethanol), stop solution (0.34 N sodium hydroxide), acid solution (3 N hydrochloric acid).

20 µL of each enzyme containing sample (total protein amounts were 2.2 pmol, 222 fmol, 22.2 fmol, 2.2 fmol, 0.2 fmol) was mixed with 980 µL working solution and incubated on a shaker at

37°C. 1 mL working solution without enzyme served as a control. 100 µL aliquots of each sample were taken in duplicates after 15, 30, 60 and 120 minutes. To each 100 µL aliquot 240 µL stop solution was added. After mixing 20 µL of OPA-solution was added. The samples were mixed and incubated for 10 minutes at room temperature. Then 40 µL of acid solution was added and after mixing the samples were centrifuged at 3000 g for 10 minutes. The supernatants were transferred to a 96-well microtiter fluorescence plate and fluorescence was measured at 365 nm extinction and 495 nm emission wavelength in a ThermoLab Fluoroscan fluorescence spectrometer. The maximum of the measured fluorescence intensity of each single enzyme dilution was determined and plotted versus the amount (mol) of ACE in the dilution.

For the MES-assay of porcine ACE a 1:10 dilution series of the enzyme in water was made with the following concentrations 20 ng/µL, 2 ng/µL, 200 pg/µL, 20 pg/µL, 2 pg/µL, 200 fg/µL and 20 fg/µL. 20 µL of each enzymes dilutions (total protein amounts were 2.2 pmol, 222 fmol, 22.2 fmol, 2.2 fmol, 0.2 fmol, 22.2 amol, 2 amol) were coupled onto CNBr- activated sepharose beads as described in 3.2.1.2. The sepharose beads with the immobilized ACE dilution were transferred into individual wells of a 96-well microtiter plate A 30 µL of 10^{-5} M solution of the reaction specific substrate angiotensin-I (Bachem) was added to each sample. The wells containing the reaction mixtures were sealed and incubated at 37 ° C in a shaker. For the mass spectrometric analysis triplicates of 1 µL aliquots were taken from the reaction mixture after 15, 30, 60, 120 minutes. The MES-assay was carried out as described in 3.2.1.2. The relative intensity of the angiotensin-II signal for each enzyme dilution during the time course dependent incubation was calculated as described in 3.2.1.2. The maximum of the measured relative angiotensin-II signal intensity of each single enzyme dilution was determined and plotted versus the amount (mol) of ACE in the dilution.

3.2.1.5 Measurement of angiotensin-II-generating activity of renal tissue in presence of protease inhibitors

The influence of inhibitors on the angiotensin-II-generating activities was examined. 50 µL of protein (approximately 1 mg) from porcine renal tissue extract was immobilized on 100 µL CNBr-activated sepharose beads as previously described in 3.2.1.2. Then the sepharose beads were divided up into five 20 µL aliquots. To each of four aliquots different protease inhibitors were added in order to reach following final concentrations:

EDTA (Merck) 1 mM, pefablock (Serva) 1 mM, angiotensin converting enzyme inactivator Cyano-Phe-Phe-OH (Bachem) 10 µM, and cathepsin inhibitor Z-Phe-Gly-NHO-Bz-pMe (Serva)

10 µM . An aliquot without inhibitor was used as control. Then to each aliquot 30 µL of 10^{-5}M angiotensin-I was added. 1 µL aliquots from the reaction mixtures were taken after 30, 60, 120, 180 and 240 minutes and the MES-assay was performed as previously described in 3.2.1.2. The maxima of the relative angiotensin-II mass signal intensities of each immobilized protein kidney extract (treated with and without protease inhibitor) were determined. The maximum of the relative intensity of the non treated aliquot was set 100 % of angiotensin-II-generating enzyme activity and the maxima of the treated aliquots were normalized. The normalized maxima of the enzyme activities were plotted versus the used inhibitors.

3.2.2 Part II - Purification of two angiotensin-II-generating enzymes from porcine renal tissue protein extract

3.2.2.1 Protein purification scouting (PPS) experiments for the determination of parameters for anion exchange chromatography

In order to find optimal parameters for the chromatographic purification of angiotensin-II-generating fractions from porcine renal tissue PPS experiments were performed using an anion exchange resin. In this experiment following parameters were varied: the pH and the concentration of NaCl in the equilibration buffer. The PPS experiments were performed in 2.2 ml deep-well microtiter plates from ABgene. The robot system Multiprobe-II-ex from Perkin-Elmer was used for liquid handling steps.

Porcine renal tissue extract was prepared by homogenization of 1 g freeze dried porcine renal tissue powder in 15 mL 10 mM phosphate buffer (pH 7.3) as described in 3.2.1.1. The Protein concentration of the resulting extract was measured by Bradford-assay and adjusted to 10 mg/ml with 10 mM phosphate buffer (pH 7.3).

For the anion -exchange chromatography PPS experiments 300 µl aliquots of Fractogel™ EMD TMAE Hicap M resin (Merck) were filled in each of 32 wells (8 × 4 matrix) of a 96-deep-well microtiter plate. Four Deep-well plates containing the compositions of the equilibration buffers were prepared according to table 3. Each buffer (40 mM) was adjusted to the pH given in brackets: N-methyl-piperazine (pH 5), Bis–Tris (pH 6), Bis–Tris–propane (pH 7), triethanolamine (pH 7.5), N-methyl-diethanolamine (pH 8), diethanolamine (pH 8.5), AMPSO (pH 9) and CAPS (pH 10). Each well contained 2 ml buffer. To each of the wells individual

amounts of NaCl were added in order to adjust the NaCl concentration according to table 2. Each gel aliquot was washed three times with 1ml of the individual buffers by copying the buffers from the equilibration-buffer deep-well plates to the deep-well plate filled with gel. After equilibration to each of the gels 1.5 ml of the equilibration buffer from the deep-well buffer plate was copied. Then aliquots of 200 µl of the renal protein extract (protein concentration: 10 mg/ml) were applied. After application of the sample the gels were washed 3 times with 600 µL of the equilibration buffers that were copied from the equilibration-buffer plate. The absorbed proteins were desorbed from the gel by eluting (3 times) with 300 µL of a 2 M NaCl-solution. Protein concentration of each resulting eluate was determined using the Bradford-assay and a 150 µL of each eluate was used for MES analysis of angiotensin-II-generating activity as described in 3.2.1.2.

NaCl				pH
0 mM	100 mM	200 mM	500 mM	
A1	A2	A3	A4	5
B1	B2	B3	B4	6
C 1	C 2	C3	C4	7
D1	D2	D3	D4	7.5
E 1	E2	E3	E4	8
F1	F2	F3	F4	8.5
G 1	G2	G3	G4	9
H 1	H2	H3	H4	10

Table 2 Scheme of the sample-application-buffer deep-well plate used for the anion exchange "F 2" for example represents a buffer with a pH of 8.5 and a sodium chloride concentration of 100 mM.

3.2.2.2 Anion exchange chromatography of porcine renal tissue protein extract

Porcine renal tissue protein extract was prepared by homogenization of 15 g of freeze-dried porcine renal tissue powder in 300 mL equilibration buffer (50 mM AMPSO pH 9) as described in 3.2.1.1. The resulting porcine renal tissue protein extract contained 7 g protein in 180 mL buffer.

320 mL of Fractogel ™ EMD TMAE 650 M (Merck) anion exchange gel was washed three times with 320 mL HPLC-grade water. Then the gel was washed 3 times with 320 mL equilibration buffer (50 mM AMPSO pH 9). 20 mL aliquots of the gel were filled into 16

numbered reaction tubes. Then the porcine renal tissue protein extract was applied to the first gel aliquot. After mixing with the resin and sedimentation the resulting supernatant was transferred to the second tube containing anion exchange gel. This sample application step was repeated with the next 14 gel containing tubes. Each of the individual gel containing tubes was washed three times with 30 mL equilibration buffer (50 mM AMPSO pH 9). For the elution of the adsorbed proteins from the gel each gel tube was mixed with 20 mL elution buffer (50 mM AMPSO, 2M NaCl, pH 9). After sedimentation of the gels the supernatants were transferred to 16 tubes. The elution was repeated twice. The protein concentration of the eluates was determined using the Bradford-assay. 50 µL of each protein containing eluate was immobilized on 100 µL CNBr-activated sepharose beads and the angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The eluates that had angiotensin-II-generating activity were characterized further in the inhibitor profiling experiment.

3.2.2.3 Inhibitor profiling of the eluates with angiotensin-II- generating activities

Each immobilized protein aliquot from the eluate of the anion exchange chromatography with angiotensin-II- generating activity was tested again for angiotensin-II-generating activity with the MES-assay in the absence (control) and presence of the protease inhibitors antipain and chymostatin. The final concentration of inhibitor was each 10 µM in 30 µL of 10^{-5}M angiotensin-I substrate solution. The sample preparation for the MES-assay was performed as previously described in 3.2.1.2. 1 µL aliquots were taken after 60, 120, 240 and 360 minutes. The maxima of the relative angiotensin-II mass signal intensities of each immobilized eluate were determined. The maxima of the relative intensity of the controls were set 100 % of angiotensin-II-generating enzyme activity and then the maxima of the inhibitor treated aliquots were normalized. The normalized maxima of the enzyme activities were plotted versus the used inhibitors.

3.2.2.4 Protein purification scouting (PPS) experiments for the determination of parameters of the intermediate chromatographic purification step

In order to find to optimal parameters for the next purification step of angiotensin-II-generating fractions from the anion exchange chromatography PPS experiments were performed using a hydroxyapatite resin and several hydrophobic resins. In this experiment the kind of

chromatographic resin was varied. The PPS experiments were performed in 2 mL Epppendorf reaction tubes.

The eluates with angiotensin-II-generating activity from fraction 6-9 of anion exchange chromatography were pooled and the protein concentration of the pool was measured using the Bradford assay. One 100 µL aliquots was 10fold diluted with equilibration buffer for hydroxyapatite chromatography (10 mM potassium phosphate pH7). Another 1mL aliquot was 10fold diluted with equilibration buffer for hydrophobic exchange chromatography (2 M NaCl, 40 mM potassium phosphate pH 7)

100 µL CHT ™ ceramic hydroxyapatite Bio-Gel (Biorad) was filled into an Eppendorf tube washed three times with 1mL HPLC-grade water. Then the gel was washed 3 times with 1 mL equilibration buffer (10 mM potassium phosphate pH 7). The diluted protein sample, containing 3 mg protein in 1 mL 10 mM potassium phosphate pH 7, was applied to the gel. After sample application the hydroxyapatite gel was 3 times with 1 mL of equilibration buffer. For the elution of the adsorbed proteins the gel was washed 3 times with 1 mL elution buffer (500 mM potassium phosphate pH 7) and the resulting eluate was collected. The protein concentration of the eluate was determined using the Bradford-assay. Aliquots of the eluates were used for the measurement of the angiotensin-II-generating activity by MES-assay as described in 3.2.1.2.

100 µL of methyl-HIC gel (Biorad), t-butyl HIC gel (Biorad), propyl EMD 650 S gel (Merck), ether 650 M gel (Tosoh), hexyl 650 C gel (Tosoh), butyl Sepharose FF gel (Amersham Biosciences), octyl Sepharose FF gel (Amersham Biosciences), phenyl Sepharose FF gel (Amersham Biosciences), isopropyl 15 ISO gel (Amersham Biosciences), phenyl 15 PHE gel (Amersham Biosciences) was filled into an Eppendorf tube and each washed three times with 1mL HPLC-grade water. Then each gel was washed 3 times with 1 mL equilibration buffer for hydrophobic exchange chromatography (2 M NaCl, 40 mM potassium phosphate pH 7). Then 100 µL of the diluted protein sample, containing 3 mg protein in 1 mL 2 M NaCl, 40 mM potassium phosphate pH 7, was applied to each of the gel aliquots. After sample application the gel aliquots were each washed 3 times with 1 mL of the equilibration buffer. For the elution of the adsorbed proteins the gel aliquots were each washed 3 times with 1 mL elution buffer (40 mM potassium phosphate pH 7) and the resulting eluate was collected. The protein concentration of the eluate was determined using the Bradford-assay. Aliquots of the eluates were used for the measurement of the angiotensin-II-generating activity by MES-assay as described in 3.2.1.2.

3.2.2.5 Hydroxyapatite chromatography

The eluates with angiotensin-II-generating activity from fraction 6-9 of the anion exchange chromatography were concentrated to a volume of 10 mL by centrifugal filtration at 2000 g with a 10 KDa cut off membrane filter unit Amicon Ultra-15 (Millipore). The protein concentrate was diluted with equilibration buffer (10 mM potassium phosphate pH 7) to 100 mL.

100 mL of CHT ™ ceramic hydroxyapatite Bio-Gel (Biorad) was washed three times with 100 mL HPLC-grade water. Then the gel was washed 3 times with 100 mL equilibration buffer (10 mM potassium phosphate pH 7). 10 mL aliquots of the gel were filled into 10 numbered reaction tubes. The protein solution (total protein amount 500 mg) was applied to the first gel aliquot. After mixing with the resin and sedimentation the resulting supernatant was transferred to the second tube containing hydroxyapatite gel. This sample application step was repeated with the next eight gel containing tubes. Each of the individual gel containing tubes was washed three times with 30 mL equilibration buffer. For the elution of the adsorbed proteins from the gel each gel tube was mixed with 10 mL elution buffer (500 mM potassium phosphate pH 7). After sedimentation of the gels the supernatants were transferred to 10 tubes. The elution was repeated twice. The protein concentration was determined using the Bradford-assay. Aliquots of the eluates were used for the measurement of the angiotensin-II-generating activity by MES-assay as described in 3.2.1.2.

3.2.2.6 Protein purification scouting (PPS) experiments for the determination of parameters of sugar affinity chromatography

In order to find to optimal parameters for the next purification step of the angiotensin-II-generating fractions from hydroxyapatite chromatography PPS experiments were performed using a lectin affinity chromatography resin. In this experiment the elution buffer was varied. The PPS experiments were performed in 2 mL Epppendorf reaction tubes.

The eluates with angiotensin-II-generating activity from fraction 1-3 of hydroxyapatite chromatography were pooled and the protein concentration of the pool was measured using the Bradford assay. One 100 µL aliquot were taken from the pool and 10fold diluted with equilibration buffer for lectin affinity chromatography (20 mM Tris-HCl, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4).

Two 500 µL aliquots of a lentil lectin sepharose 4B (Amersham Biosciences) resin was filled into Eppendorf tubes and washed three times with 1mL HPLC-grade water each. Then each of

the gel aliquots were washed 3 times with 1 mL equilibration buffer (20 mM Tris-HCl, 500 mM NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4). 500 µL aliquot of diluted protein sample, each containing about 100 µg total protein amount, were applied to the prepared aliquots of the lentil lectin sepharose resin. After sample application the lentil lectin sepharose resin was washed 3 times with 1 mL of equilibration buffer. For the elution of the adsorbed proteins the first gel aliquot was washed 3 times with 1 mL elution buffer 1 (20 mM TRIS-HCl, 250 mM Methyl-α-D-glycopyranoside, pH 7.4) and the resulting eluate was collected. The second gel aliquot was washed 3 times with 1 mL elution buffer 2 (20 mM TRIS-HCl, 250mM Methyl-α-D-mannopyranoside, pH 7.4) and the resulting eluate was collected. The protein concentrations of both the eluates were determined using the Bradford-assay. Aliquots of the eluates were used for the measurement of the angiotensin-II-generating activity by MES-assay as described in 3.2.1.2.

3.2.2.7 Lectin affinity chromatography

The eluates from the hydroxyapatite chromatography with angiotensin-II-generating activity were concentrated to a volume of 3 mL by centrifugal filtration at 2000 g with a 10 kDa cut off membrane filter unit Amicon Ultra-15 (Millipore). The protein concentrate was diluted with equilibration buffer (20 mM Tris-HCl, 500 mM NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4) to 30 mL. The total protein amount was 100 mg.

6 mL of lentil lectin sepharose 4B (Amersham Biosciences) was filled into an empty XK 16/20 column (Amersham Biosciences). The column was assembled into the ÄKTA purifier (Amersham Biosciences) HPLC-system. The column was washed first with water then with equilibration buffer until the $UV_{280\ nm}$ signal reached zero. The sample was applied via a sample pump at a constant flow rate of 0.5 mL/min. The biomolecules were removed with equilibration buffer until the $UV_{280\ nm}$ signal was stable. The adsorbed proteins were eluted from the gel with a linear gradient of five column volumes (CV) from 0% to 100% elution buffer (20 mm TRIS-HCl, 250 mM Methyl-α-D-glycopyranoside, pH 7.4). Then the column was washed with three CV of 100 % elution buffer. Fractions of 10 mL volumes were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The fractions that had angiotensin-II-generating activity were characterized further in the inhibitor profiling experiment.

3.2.2.8 Inhibitor profiling of the eluates from lectin affinity chromatography with angiotensin-II- generating activities

Eluates from all weakly binding fractions of the lectin affinity chromatography were pooled as well as the eluates from the binding fraction. Each pool was tested again for angiotensin-II-generating activity with the MES-assay in the absence (control) and presence of the following protease inhibitors. antipain 10 µM final concentration, chymostatin 10 µM final concentration, angiotensin converting enzyme inactivator Cyano-Phe-Phe-OH (Bachem) 10 µM final concentration, EDTA 1mM final concentration, aprotinin 50 µg/mL. The maxima of the relative angiotensin-II mass signal intensities of each immobilized eluate (control and inhibitor treated) were determined. The maxima of the relative angiotensin-II mass signal intensities of the controls were set to 100 % and the maxima of the relative angiotensin-II mass signal intensities of the treated aliquots were normalized. The enzyme activities were plotted versus the used inhibitors.

3.2.2.9 Chymostatin-Antipain affinity chromatography

Angiotensin-II-generating protein fractions from the lectin affinity chromatography experiment that were inhibited by chymostatin and antipain were pooled and applied to a chymostatin-antipain chromatography. The pooled fractions were concentrated to a volume of 0.5 mL by centrifugal filtration at 2000 g with a 10 kDa cut off membrane filter unit Amicon Ultra-15 (Millipore). The protein concentrate was diluted with equilibration buffer (50 mM HEPES, 500 mM NaCl, pH 8) to 5 mL. Total protein amount was 5 mg.

For the preparation of the chymostatin-antipain-inhibitor gel the ligands chymostatin and antipain were covalently coupled via their carboxyl group to the gel matrix in the presence of the coupling agent carbodiimide. 10 mL of EAH-Sepharose 4B (Amersham Biosciences) were washed three times first with 500 mM NaCl then with 100 µM HCl pH 4.5. 10 mL of a solution of 1 mM antipain and 1 mM chymostatin in 100 µM HCl pH 4.5 was prepared. 400 mg of EDC (N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride) was mixed with 10 mL of the prepared EAH-Sepharose gel and 10 mL of the 1mM antipain/chymostatin solution. The mixture was incubated over night on an overhead rotor at 4° C. In the first two hours the pH was checked regularly and adjusted to pH 4.5 with NaOH. After incubation the sepharose beads were sedimented and the supernatant was removed. The sepharose beads were washed three times alternately with 0.1 M acetic acid (in 500 mM NaCl, pH 4) and 0.1 M TRIS (in 500 mM NaCl,

pH 8) in order to remove the non coupled ligands. At last the gel was washed extensively with HPLC-grade water.

5 mL of the prepared gel was filled into an empty C10/10 column (Amersham Biosciences). Then the column was assembled into the ÄKTA purifier (Amersham Biosciences) HPLC-system. The column was washed first with water then with equilibration buffer (50 mM HEPES, 500 mM NaCl, pH 8) until the $UV_{280\ nm}$ signal was stable. The protein sample was applied to the column with a constant flow rate of 0.5 mL/min. The unbound biomolecules were removed with equilibration buffer until the UV signal was stable. The bound proteins were desorbed from the gel with an isocratic gradient from 0% to 100% elution buffer (50 mM potassium phosphate pH 4). The elution was continued until the final pH 4 was reached and the $UV_{280\ nm}$ signal reached zero. Fractions of ten mL volumes were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The fraction with angiotensin-II-generating activity was named Fraction I and applied to SDS-PAGE (PART IV-protein identification experiments).

3.2.2.10 Size exclusion chromatography

The binding fraction of the lectin affinity chromatography that showed an inhibition of their angiotensin-II-generating activities in presence of EDTA and ACE- specific inactivator were pooled and applied to a size exclusion chromatography. The pooled fractions were concentrated to a volume of 50 µL by centrifugal filtration at 2000 g with a 10 kDa cut off membrane filter unit Amicon Ultra-5 (Millipore). The protein concentrate was diluted with running buffer (50 mM potassium phosphate, 150 mM NaCl, pH 7) to 250 µL. Total protein amount was 5 mg.
The Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences) had a total bed volume of 24 mL. The void volume was 7.7 mL. The column was assembled into the ÄKTApurifier (Amersham Biosciences) automated HPLC-system. The column was first washed with water and then with running buffer (50 mM potassium phosphate, 150 mM NaCl, pH 7). A run with a mixture of standard proteins and peptides was done in order to prepare a calibration curve of the partition coefficient K_{av} versus the molecular weights of the standard proteins and peptides. Therefore 250 µL of a mixture of blue dextran (1mg/mL), Thyroglobulin (2.5 mg/mL), Ferritin (0.7 mg/mL), human Immunoglobulin G (3 mg/mL), bovine serum albumin (2 mg/mL), β-lactoglobulin (2mg/mL), cytochrome C (2 mg/mL), and vitamin B12 (0.5 mg/mL) was applied to the column. The constant flow was set 0.5 mL/min. The sample molecules were all separated

and eluted after the total volume of 24 mL of the running buffer. The retention volumes of each standard molecule were determined and the partition coefficients calculated. The partition coefficients were plotted versus the decimal logarithm of the corresponding molecular weights and a calibration function was calculated. After the run of the standard molecules the sample protein solution was applied to the column. The same parameters as for the standard run were used for the separation of the sample protein solution. Fractions of 1 mL volume were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. By using the determined retention volumes the partition coefficients K_{av} of the separated sample proteins were calculated. The approximate molecular weights of the different angiotensin-II-generating fractions were calculated with the assistance of the calibration function. The fraction with angiotensin-II-generating activity was named Fraction II and passed to two-dimensional electrophoresis (Part IV-protein identification experiments).

3.2.3 Part III- Purification of an angiotensin-II-generating fraction from porcine renal tissue protein extract

3.2.3.1 Anion exchange chromatography of porcine renal tissue protein extract

4 g of freeze-dried porcine renal tissue powder was homogenized in 40 mL equilibration buffer (50 mM AMPSO, 1 mM EDTA, pH 8.3) and processed as described in 3.2.1.1. The resulting porcine renal tissue protein extract contained 3 g protein in 30 mL buffer.

100 mL of Fractogel ™ EMD DEAE 650 M (Merck) anion exchange gel was washed three times with 100 mL HPLC-grade water. The anion exchange gel was filled into an empty XK 50/30 column (Amersham Biosciences). Then the column was assembled into the ÄKTA purifier (Amersham Biosciences) HPLC-system. The column was washed with equilibration buffer until the $UV_{280\ nm}$ signal reached zero. The sample was applied via a sample pump at a flow rate at a constant flow rate of 1 mL/min. The unbound biomolecules were removed by washing with equilibration buffer until the $UV_{280\ nm}$ signal was stable. The adsorbed proteins were eluted from the gel with a linear gradient of three column volumes (CV) from 0% to 100% elution buffer (50 mM AMPSO, 1 mM EDTA, 1 M NaCl, pH 8.3). The column was washed with four CV of 100 % elution buffer. Fractions of 40 mL volumes were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The fractions with angiotensin-II-generating activity were applied to wheat germ lectin chromatography.

3.2.3.2 Wheat Germ Lectin chromatography

The eluate from the anion exchange chromatography with A-II-generating activity (elution volume from 435-475 ml) was concentrated to a volume of 3 mL by centrifugal filtration at 2000 g with a 10 kDa cut off membrane filter unit Amicon Ultra-15 (Millipore). The protein concentrate was diluted with equilibration buffer (20 mM TRIS-HCl, 500 mM NaCl, pH 7.4) to 30 mL. Total protein amount was 10 mg.

10 mL of wheat germ lectin sepharose 4B (Amersham Biosciences) was filled into an empty XK 16/20 column (Amersham Biosciences). Then the column was assembled into the ÄKTA purifier (Amersham Biosciences) HPLC-system. The column was washed first with water then with equilibration buffer until the $UV_{280\ nm}$ signal was zero. The sample was applied via a sample pump at a flow rate at a constant flow rate of 1 mL/min. The unbound biomolecules were removed by washing with equilibration buffer until the $UV_{280\ nm}$ signal was stable. Then the adsorbed proteins were eluted from the gel with an isocratic step from 0% to 100% elution buffer (20 mM TRIS-HCl, 300 mM N-acetyl-glucosamine pH 7.4). Then the column was washed with five CV of 100 % elution buffer). Fractions of 10 mL volumes were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The fractions that had angiotensin-II-generating activity were characterized further in the inhibitor profiling experiment.

3.2.3.3 Inhibitor profiling of the eluates with angiotensin-II- generating activities

Each immobilized eluate of the wheat germ lectin chromatography with angiotensin-II-generating activity was tested again for angiotensin-II-generating activity with the MES-assay in the absence (control) and presence of the following protease inhibitors: antipain 10 µM final concentration, chymostatin 10 µM final concentration and aprotinin 50 µg/mL final concentration each in 30 µL of a 10^{-5}M angiotensin-I substrate solution. The sample preparation for the MES-assay was performed as previously described in 3.2.1.2. Therefore 1 µL aliquots were taken after 60, 120, 240 and 360 minutes. The maxima of the relative angiotensin-II mass signal intensities of each immobilized eluate (control, inhibitor treated) were determined. The maxima of the relative intensity of the non treated aliquot were set 100 % of angiotensin-II-generating enzyme activity and then the maxima of the treated aliquots were normalized. The angiotensin-II-generating enzyme activities were plotted versus the used inhibitors.

3.2.3.4 Aprotinin affinity chromatography

Protein fractions from the wheat germ lectin chromatography with angiotensin-II-generating activities were pooled. The pooled fractions were concentrated to a volume of 0.5 mL by centrifugal filtration at 2000 g with a 10 kDa cut off membrane filter unit Amicon Ultra-15 (Millipore). The protein concentrate was diluted with equilibration buffer (50 mM HEPES, 500 mM NaCl, pH 8) to 5 mL. The total protein amount was 0.5 mg.

For the preparation of the aprotinin-inhibitor gel the protease inhibitor aprotinin was covalently coupled via its amino group to the gel matrix. 5 mL of CNBr-activated sepharose 4B (Amersham Biosciences) were washed three times first with water then with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). 20 mg of aprotinin was dissolved in 5 mL of coupling buffer and the solution was added to the washed sepharose gel. The mixture was incubated for two hours at room temperature on an overhead rotor. After the coupling of aprotinin the free binding sites of the sepharose beads were blocked with 5 mL blocking buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, 0.2 M glycine pH 8.3) for 2 h at room temperature. After blocking the beads were washed four times with HPLC-grade water.

The prepared gel (5 mL) was filled into an empty C10/10 column (Amersham Biosciences). Then the column was assembled into the ÄKTA purifier (Amersham Biosciences) HPLC-system. The column was washed with equilibration buffer (50 mM HEPES, 500 mM NaCl, pH 8) until the $UV_{280\ nm}$ signal was stable. The protein sample was applied to the column with a constant flow rate of 0.5 mL/min. The unbound biomolecules were removed by washing with equilibration buffer until the UV signal was stable. The bound proteins were desorbed from the gel with an isocratic step from 0% to 100% elution buffer (50 mM potassium phosphate pH 3). The elution was continued until pH 3 was reached and the $UV_{280\ nm}$ signal was stable. Fractions of five mL volumes were collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2. The fraction with angiotensin-II-generating activity was named Fraction III and applied to SDS-PAGE (Part IV-protein identification experiments).

3.2.3.5 Determination of the molecular weight of the Fraction III

250 µL of the eluate containing purified Fraction III was applied to a size exclusion chromatography. The experiment was carried out on Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences) as described in 3.2.2.10. Fractions of 0.5 mL volume were

collected. The angiotensin-II-generating activity of the eluates was measured by the MES-assay as described in 3.2.1.2.

3.2.3.6 Test on substrate specifity of Fraction III

For the detection of ACE-like activity with the MES-assay aliquots of the immobilized Fraction III were incubated with 30 µL of 10^{-5} M angiotensin-I. For the detection of renin-like activity with the MES-assay aliquots of the immobilized purified Fraction III were incubated with 10^{-5} M porcine renin tetradecapeptide substrate in presence and absence of 10 µM of a renin specific inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe (Bachem).For the detection of kallikrein-like activity with the MES-assay aliquots of the immobilized purified Fraction III were incubated with a L-kininogen analogue 10^{-5}M Lys-Bradykinin-Ser-Val-Gln-Val-Ser (Bachem). For detection of kallidin degrading activities MES-assay aliquots of the immobilized purified Fraction III were incubated with 10^{-5}M Kallidin (synonymous Lys-Bradykinin) (Bachem).

3.2.3.7 Database matching of the retrieved substrate specifity information of Fraction III

The BRENDA enzyme database (http://www.brenda.uni-koeln.de) was searched using the “advanced search” service. In the entry mask the button “search in text fields” with the sub item “natural product” was enabled and the term “angiotensin II” or “desArg^{10}-kallidin” or “kallidin” was entered and the search was committed.
Further the Merops peptidase database (http://merops.sanger.ac.uk/) was searched using the “searches” service. The options “search by specifity” and “How may this substrate be cleaved?” were enabled and “angiotensin I” or “renin decapeptide substrate” were selected from the dropdown list und the search was submitted. Merops returned the query substrate with the possible cleavage sites mapped on it and a table of cleavage site positions. Since there was no entry in the dropdown list for the Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate another attempt was done using the option “What peptidase can cleave this bond?” in the “searches” service of the Merops database. In the entry mask the given amino acid sequence and the cleavage site of the substrates (angiotensin-I, renin tetradecapeptide substrate, and Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate) were entered und the search submitted. The Merops database then returned with the appropriate peptidases that are able to cleave at the submitted cleavage site of the substrates.

3.2.4 PART IV- Protein identification experiments of Fraction I, II, and III

3.2.4.1 Separation of proteins by gel electrophoresis

Gel electrophoresis of the purified protein fractions was performed at the WITA GmbH. Fraction I and III were subjected to a one-dimensional electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were concentrated using ethanol precipitation and the disulfide bonds of the proteins were reduced using 1, 4-Dithio-DL-threitol (DTT). Then both samples were separated on a 10 % acrylamide/ bisacrylamide SDS-PAGE according to the WITA protocols (50).

Fraction II was precipitated with ethanol and reduced using DTT. Then it was subjected to a high-resolution two-dimensional- electrophoresis by combining isoelectric focussing (IEF, first dimension) and sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE, second dimension) as described by Klose and Kobalz (51).

3.2.4.2 In- gel digestion of protein bands with specific endopeptidases

All gels were stained with Coomassie Brilliant Blue G-250. Gels were scanned and protein bands were detected. The respective protein bands were excised and minced into $1mm^3$ pieces and transferred into a clean 0.5 mL reaction tube. Afterwards the gel pieces were each washed and reduced using 200 µL of a 200 mM ammonium bicarbonate/ 10 mM DTT buffer. The gel pieces were destained by incubation for 15 min in 50 % acetonitrile, 50 mM ammonium bicarbonate solution. The solution was discarded and the gel pieces were dehydrated by incubation for 20 min in 100 % acetonitrile. Then the acetonitrile solution was removed and the gel pieces were completely air-dried.

The endopeptidase digestion solutions (trypsin and V8-protease) were prepared as follows:

20 µg of lyophilized modified sequencing grade trypsin (Promega) was resuspended in 20 µL of 50 mM acetic acid yielding a 1 µg/µL stock. The stock solution was diluted to 1µg/50 µL with digestion buffer I (50 mM ammonium bicarbonate, pH 8). 25 µg of lyophilized sequencing grade V8-protease was resuspended in 25 µL HPLC-grade water yielding a 1µg/µL stock. The stock solution was diluted to 1µg/50 µL with digestion buffer II (50 mM potassium phosphate, pH 7.8).

The dried gel piece containing the band from the Fraction II was rehydrated by adding 50 µL of the diluted trypsin solution (in digestion buffer I). The dried gel pieces containing the band from the Fraction I and III respectively were rehydrated by adding 50 µL of the diluted V8-protease

solution (in digestion buffer II). The digestion was carried out over night at 37 °C with gently agitation. After digestion the supernatants (containing the proteolytic peptides) were removed to a clean 0.5 mL reaction tube and 50 µL of extraction solution (60 % acetonitrile, 1% TFA) was added to the gel pieces. After 10 min the supernatants (containing additional proteolytic peptides) were removed and transferred to the reaction tube containing the proteolytic peptides. The extraction was repeated twice. The pooled supernatants were dried by centrifugal evaporation in a speed-vac for 30 minutes.

3.2.4.3 Peptide mass fingerprint

The proteolytic peptides from Fraction I, II and III were subjected to a peptide mass fingerprint. Therefore an aliquot of the proteolytic peptides from the in-gel digestion was desalted using C18-Zip tips (Millipore). After desalting 1 µL of the sample was mixed with 1 µL of MALDI-TOF matrix solution (20 mg/ mL α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile, 0.1% trifluoroacetic acid in water) and spotted on a 384-MPT AnchorChip target (Bruker). The spots were washed with HPLC-grade water and recrystallized.
Afterwards the target was introduced into a Bruker Reflex III MALDI-TOF mass spectrometer and positively charged ions were analyzed using the reflector mode and delayed ion extraction. The spectra were recorded with a 2-GHz sampling rate, 150 ns extraction delay time and deflection was used to suppress ions smaller than 400 m/z and bigger than 4000 m/z. A nitrogen laser with an emission wavelength of 337 nm and 3 ns and 3-ns pulse was used. For each sample, 100 single-shot spectra were accumulated, which result from 10 different spots per sample (10 spectra per spot). The data processing and analysis was performed automatically using the Reflex III software package XMASS 5.1, Biotools 2.2 and Mascot. Automated peak picking was performed using the SNAP algorithm in order to label the first monoisotopic peak of the peptide mass signal. The MS-Picky software and method of Johan Gobom (52) was used for the external calibration of the device. For the internal calibration the autolytic peptides from trypsin and V-8 proteases were used.

3.2.4.4 NanoLC-ESI-MS/MS

The proteolytic peptides from Fraction I and III were subjected to nanoLC-ESI-MS/MS. These experiments were performed at the WITA GmbH. An aliquot of the proteolytic peptides from the in-gel digestion was desalted using C18-Zip tips (Millipore). After desalting the samples were

dried and resuspended in 5 µL 0.2 % TFA. 4 µL of the sample was injected to a nanoLC-ESI-MS/MS. The HPLC-system was from Ultimate ™. The column for the separation of the peptides was a high resolution C18 Reversed phase column. The eluates from the column were applied to the Esquire HCT ESI mass spectrometer from Bruker Daltonics™. A MS^2 analysis of the peptide peaks was performed. The further data processing and analysis was performed automatically using the software Hystar 2.3, Esquire control 5.1, HyStarPP 2.3., DataAnalysis 3.1., Biotools 2.2 and Mascot.