5. Discussion

The renin- angiotensin-system (RAS) is the main system responsible for the regulation of arterial blood pressure. The octapeptide angiotensin-II is the physiologically active hormone of the renin-angiotensin-system. Angiotensin-II is formed by a proteolytic cleavage of the prohormone angiotensin-I. For a long time the dipeptidyl-peptidase angiotensin converting enzyme (ACE) was considered to be the main enzyme to convert angiotensin-I to angiotensin-II. Lately ACE-independent ways of angiotensin-II formation became the focus of cardiovascular research. Several studies could prove the existence of several ACE-independent angiotensin-II-generating enzymes.

Therefore the object of this work was the purification, characterization and identification of angiotensin-II-generating enzymes from porcine renal tissue. This work comprises four general steps. First an assay system for the detection of angiotensin-II-generating enzyme activities was established. Second a strategy for the purification of the angiotensin-II-generating enzymes was developed. This purification strategy was controlled by the assay system for the detection of angiotensin-II-generating enzyme activities. Third the angiotensin-II-generating enzyme fractions from porcine renal tissue were purified near homogeneity. Fourth the purified angiotensin-II-generating enzyme fractions were identified and characterized.

Conventional assay systems for the detection of angiotensin-II-generating enzyme activities either use synthetic fluorescence or chromogenic labelled substrates (53, 54), radioactive labelled substrates (55), or natural substrates which enzymatic reaction products are monitored using analytical HPLC (56). Synthetic fluorescence or chromogenic labelled substrates are not identical with the original substrate and may alter enzyme kinetics (57). The use of radioactive substrates evades this problem but the preparation and use of radioactive substrates is expensive and hazardous. The use of natural substrates such as angiotensin-I and the detection of their reaction products using analytical HPLC give no clear verification of the identity of the reaction products.

In order to circumvent these problems a new assay system for the detection of angiotensin-II-generating enzymes was established-the MES-assay system. Therefore the proteins from porcine renal tissue and porcine angiotensin converting enzyme (ACE) were covalently immobilized to activated affinity beads. The immobilized proteins were incubated with angiotensin-I, the natural

substrate for the detection of angiotensin-II generating enzyme activities. After particular incubation times aliquots of the reaction mixtures were taken and analyzed by MALDI-TOF mass spectrometry. As expected the incubation of porcine ACE with angiotensin-I yielded angiotensin-II as reaction product as shown in figure 10. The incubation of immobilized proteins from porcine renal tissue with angiotensin-I yielded the reaction products angiotensin-I and angiotensin (1-7) as shown in figure 10. The identities of the products were confirmed by their m/z ratios-1047 for angiotensin-II and 900 for angiotensin (1-7).

The MES-assay system proved to have several advantages compared to conventional assay systems. Contrary to conventional assay system false positive results are avoided since the authentic active site specific substrates are used. The fate of the substrates is controlled-eventually occurring side products are easily monitored with the mass spectrometry.

The MES-assay achieved a high detection limit of ACE as shown in figure 12 b. Compared to a fluorometric enzyme assay (figure 12a) the MES-assay was 1000fold more sensitive. Because even amol amounts of ACE were detected the MES-assay is sensitive enough to detected little amounts of angiotensin-II-generating enzyme activities.

A challenge of the MES-assay is the quantification of the reaction products by mass spectrometry (58). In order to purify angiotensin-II-generating enzyme fractions from porcine renal tissue fractions with high enzymatic activity have to be distinguished from fractions with low activity. Therefore the measured MES angiotensin-II signal intensities have to correlate with the enzyme activities of the sample. Previously a quantitative analysis using MALDI-TOF MS of low molecular mass substrates and products of an enzyme-catalyzed reaction was demonstrated by Kang et al. (59) using internal standards.

Within this work it was shown that the obtained MES angiotensin-II signal intensities show a correlation between enzyme activities and MES-signal intensities as shown in figure 11a and 11b. Porcine angiotensin converting enzyme showed a high angiotensin-II-generating activity in absence of a specific inactivator (figure 11a and b). In presence of a specific inactivator the angiotensin-II-generating activity of ACE should be lower. As expected the relative angiotensin-II-generating activity in presence of the inactivator (measured by the MES-assay) was much lower than measured activity in absence of the specific inactivator (diagram figure 11a).

Enzyme fractions with high angiotensin-II-generating activity can be distinguished from those with low angiotensin-II-generating activity with the use of the MES-assay system.

The precision of the MES-assay is sufficient enough to study the effect of inhibitors on angiotensin-II-generating activities of complex protein fractions as shown in figure 13. The

metallo protease inhibitor EDTA, the serine protease inhibitor Pefablock and the ACE-specific inactivator reduced the angiotensin-II-generating activity of porcine renal tissue protein extract. Therefore protein extract from porcine renal tissue must contain several types of angiotensin-II-generating proteases. In presence of a specific ACE-inactivator the angiotensin-II-generating activity is not completely blocked but reduced to 50 % of the activity in absence of the inactivator. Therefore porcine renal tissue protein extract must have ACE-independent angiotensin-II-generating activities.

A fast and reliable strategy for the purification of the target angiotensin-II-generating enzymes has to be developed. The aim of the purification is the separation of the angiotensin-II-generating enzyme fractions from other proteins of the tissue extract and the concentration of the angiotensin-II-generating enzyme fractions. A common tool for the purification of proteins is liquid chromatography. Liquid chromatography is a purification that maintains the enzymatic activities of a protein during the course of purification. Because porcine renal tissues extract contains a high amount of proteins a chromatography purification method with high binding capacity is needed for the first purification is needed. Ion exchange chromatography is a liquid chromatography with high binding capacity (60). In order to find optimal purification parameters protein purification scouting (PPS) experiments were performed. PPS experiments using anion exchange chromatography and porcine renal tissue protein extract as a sample and yielded a protein fraction with high angiotensin-II-generating activity with an equilibration buffer at pH 9 and 0 mM NaCl as shown in table 4. Porcine renal tissue protein extract was applied to an anion exchange chromatography using the determined parameters and protein fractions with angiotensin-II-generating activity were obtained. The chromatography was done in sample displacement mode. Sample displacement mode is capable to efficiently separate a target protein from other major impurities and to utilize the full binding capacity of the chromatography media (42). After the first chromatographic purification step further purification steps were necessary. In order to find optimal purification parameters PPS experiments were performed with the active fractions of the anion exchange chromatography prior to each chromatographic purification step. A combination of hydroxyapaptite chromatography (figure 16) and lentil lectin chromatography (figure 17) led to two different angiotensin-II-generating enzyme fractions. As shown in figure 17 the fraction that eluted first (fraction A) from the lentil lectin chromatography bound weakly to the lentil lectin resin; the angiotensin-II-generating enzyme in this fraction contained no glucose type polysaccharide chain as post translational modification. The second fraction (fraction B) had a high affinity to the lentil lectin resin and was eluted with a competitive glucose

type sugar gradient as shown in figure 17. Therefore the enzymes in this fraction must contain glucose type polysaccharide chains as post translational modification.

Further inhibitor profiling experiments were performed to characterize the angiotensin-II-generating activities of the two chromatographic fractions. The inhibition profiling experiments showed an individual inhibitor profile for each fraction as shown in figure 18. Fraction B showed an inhibition of its angiotensin-II-generating activity in presence of the EDTA and ACE-specific inactivator. Fraction A showed an inhibition of its angiotensin-II-generating activity in presence of antipain and chymostatin. An inhibitor affinity resin was produced by the covalent coupling of chymostatin and antipain to a chromatography resin. As for the last chromatographic purification step fraction A of the lentil lectin chromatography was applied to the chymostatin-antipain chromatography. One fraction with angiotensin-II-generating activity was eluted (figure 19). This fraction (named Fraction I) was passed to the protein identification experiments.

Fraction B of the lentil lectin chromatography was applied to size exclusion chromatography. As shown in figure 20 one fraction with angiotensin-II-generating activity was eluted from size exclusion chromatography. The molecular weight of the angiotensin-II-generating enzyme in this fraction was approximate 200 kDa (figure21). The angiotensin-II-generating fraction from size exclusion chromatography was named Fraction II and passed to the protein identification experiments.

In order to purify further angiotensin-II-generating enzymes a second purification strategy was planned and performed. This purification strategy was inspired by an article by Sasaguri et al. (61). Sasaguri et al. purified a 65 kDa kinin- and angiotensin-II-forming enzyme from dog heart by a combination of anion exchange chromatography, wheat germ lectin chromatography and aprotinin affinity chromatography. He was not able to obtain the amino acid sequence of this enzyme and protein identification experiments failed (62). He proved the presence of this enzyme. in kidney by using western blot analysis. The same combination of anion exchange chromatography (figure 22), wheat germ lectin chromatography (figure 23) and aprotinin affinity chromatography (figure 25) was used for the purification of further angiotensin-II-generating-enzyme fraction. Porcine renal tissue protein extract was applied to anion exchange chromatography column and an angiotensin-II-generating fraction was eluted from the column with an increasing gradient of NaCl (figure 22). The angiotensin-II-generating fraction from the anion exchange chromatography was applied to wheat germ lectin chromatography. A fraction with angiotensin-II-generating activity was eluted form the wheat germ lectin column using the competitive sugar N-acetyl glucosamine (figure 23). Thus the enzyme responsible for

angiotensin-II-generating activity of this fraction from wheat germ lectin chromatography must contain N-linked polysaccharide chains as post translational modifications. Inhibitor profiling experiment using the angiotensin-II-generating fraction from wheat germ lectin chromatography showed an inhibition of its enzyme activity in presence of aprotinin, an inhibitor of serine protease, but no inhibition in presence of chymostatin or antipain (figure 24). This feature was used for the last purification step of the wheat germ lectin binding fraction using aprotinin as a ligand for inhibitor affinity chromatography (figure 25). Using aprotinin inhibitor affinity chromatography the angiotensin-II-generating enzyme was purified near homogeneity, named Fraction III and passed to the protein identification experiments.

The Fraction I, II and III were applied to the protein identification experiments. Therefore the protein molecules of the samples were separated using electrophoresis. Fraction I (figure 30) and Fraction III (figure 31) were applied to SDS-PAGE. Fraction II was subjected to two-dimensional electrophoresis (figure 32). The protein bands or spots resulting from electrophoresis were excised and digested with specific endopeptidases and applied to peptide mass fingerprint experiments. Using peptide mass fingerprint the enzymes responsible for the angiotensin-II-generating activity of Fraction II and III could not be identified. There might be several reasons for the unsuccessful identification using the fingerprint method: 1. an underestimated mass measurement error, 2. a non-specific cleavage of the protein sample by the endopeptidase, 3. unsuspected chemical and post-translation modification of the sample protein and at last the protein sequence is not in the database (63). In general peptide mass fingerprint has a small error tolerance since the information used for the database matches are merely the molecular weights of the proteolytic peptides.

The 45 kDa protein band of the SDS-PAGE of Fraction I (figure 30) was identified as porcine cathepsin G (figure 32) using peptide mass fingerprint. The complete sequence of porcine cathepsin G is not included in protein sequence databases yet. Human cathepsin G has about 73 % sequence homology to the partially known sequence of Cathepsin G from pig. Human cathepsin G is a single chain glycoprotein with a molecular weight of about 29 kDa. Cathepsin G is a neutrophil serine protease and belongs to the family of endopeptidase with chymotrypsin-like activity i.e. its sequence has a strong a strong similarity to trypsin and chymotrypsin (64). Cathepsin G is able to convert angiotensin-I to angiotensin-II and to generate angiotensin-II directly from angiotensinogen in vitro (65). The identification of the enzyme responsible for the angiotensin-II-generating activity of Fraction I as cathepsin G is plausible. The angiotensin-II-

generating activity of Fraction I was inhibited in presence of antipain and chymostatin (figure 18). Antipain and chymostatin are both inhibitors of serine and cysteine proteases. Human cathepsin G is inhibited in presence of chymostatin (66). Mouse cathepsin G is inhibited in presence of antipain (67). The angiotensin-II-generating activity of Fraction I bound weakly to the lentil lectin chromatography resin (figure 17), therefore it contains no glucose type polysaccharide chains as post translational modifications. Human Cathepsin G contains one potential N-glycosylation site and three disulfide bridges (68).

Cathepsin G is a potential part of the locally expressed angiotensin-II-generating enzymes. Currently Cathepsin G was found to be locally expressed in the arterial wall (69). It has been found in high concentrations in the human neutrophil azurophil granules (26) and localized in granules of pro inflammatory monocytes with peroxidase activity (70). The enzyme plays a role in the proteolytic degradation of connective tissue proteins and plasma proteins during inflammation (71). Recent studies provided evidence that neutrophil serine proteases are able to activate specifically pro-inflammatory cytokines (72). According to mRNA-databases cathepsin G is mainly expressed in CD34 cells and in bone marrow but in kidney only a low expression was measured (73). In immunohistochemically stained tissue sections using cathepsin G specific antibodies high concentrations of the enzyme were detected in the bone marrow and in macrophages but no staining was detected in the kidney (74, 75). The question is why cathepsin G was detected in kidney in this work but not detected with immunohistological staining. The antibody used for the immunohistochemical staining of cathepsin G in tissue was a monoclonal antibody from mouse (76). The recognized epitope might be hidden in case that cathepsin G is bound to another protein such as native inhibitors such as the secretory leucocyte proteinase inhibitor (SLPI). SLPI is the predominant physiologic inhibitor of cathepsin G (77) and it was shown to be present in the renal tubuli cells of the kidney (78). This observation was confirmed by the immunohistochemically stained tissue sections using SLPI specific antibodies (75). Forming complexes of cathepsin G with its native inhibitors may hide the epitope for its recognition by cathepsin G specific antibodies and therefore lead to a negative result the immunohistochemically staining of the enzyme in kidney tissue.

Besides the role of cathepsin G in inflammation, this enzyme has not been shown to produce angiotensin-II in tissue. Previously angiotensin-II was considered as a circulating hormone playing a major role in the regulation of blood pressure. Now there is increasing evidence that angiotensin-II is also capable of inducing inflammation (79, 80). Local angiotensin-II has been

shown to be more closely related to inflammation than circulating angiotensin-II (81). Non-ACE pathways including cathepsin G, chymase, tonin have been suggested to produce angiotensin-II in tissue (82). Angiotensin-II induces inflammation mainly via the AT_1-receptor (83). Within this work it is shown for the first time that cathepsin G contributes to the angiotensin-II production in porcine kidney. Some of porcine kidneys obtained from the slaughterhouse might have been in a condition of inflammation.

The proteolytic peptides of the protein spots 4 and 5 from the two-dimensional electrophoresis of Fraction II were analyzed by nanoLC-ESI-MS/MS. The spot 4 and 5 from the two-dimensional electrophoresis of Fraction II were identified by homology as angiotensin converting enzyme from rabbit (figure 33). Porcine angiotensin converting enzyme is not included in the Uniprot protein database yet. Angiotensin converting enzyme is a metallo protease and its angiotensin-II-generating activity is inhibited by EDTA (84) and ACE-specific inactivator (85). Angiotensin converting enzyme was purified from porcine kidney, its molecular weight was estimate 195 kDa (determined by size exclusion chromatography) and it contains about 8% per weight polysaccharides as post translational modifications (86). These findings are consistent with the experimental data. The angiotensin-II-generating activity of Fraction II was inhibited in presence of the metallo protease inhibitor EDTA and ACE-specific inactivator (figure 18). The molecular weight determined by size exclusion chromatography was approximately 200 kDa (figure 21). Furthermore the angiotensin-II-generating activity of this fraction bound to the lentil lectin chromatography resin, therefore must contain glucose type polysaccharide chains as post translational modification (figure 17).
ACE was already purified from porcine kidney (86). The identification of angiotensin converting enzyme meets the expectations and proves that the strategy for the purification and identification of angiotensin-II-generating enzymes from porcine kidney protein extract was successful. It is the “proof of principle” that the MES-assay system and purification strategy guided by the PPS-system leads to a successful purification and identification of a target enzyme.

The proteolytic peptides from the high molecular weight protein band from the SDS-PAGE of Fraction III were analyzed by nanoLC-ESI-MS/MS. The derived amino acid sequences (table 9) did not match to a protein database entry. The genome of the pig is not completely sequenced yet. Therefore pig protein sequence entries within a protein databases such as Uniprot are incomplete. If there are no homologies of the derived porcine sequences of the proteolytic peptides to other organisms the identification fails.

Further characterization experiments concerning the molecular weight of the angiotensin-II-generating enzyme of Fraction III and the substrate specifity were performed. For the determination of the molecular weight of the angiotensin-II-generating enzyme in Fraction III size exclusion chromatography was performed (figure 26). As shown in figure 27 the estimated molecular weight of the angiotensin-II-generating enzyme in Fraction III was approximately 160 KDa. The chromatogram of the size exclusion experiment of Fraction III (figure 26) also shows that Fraction IIII probably contains still more than one protein.

Fraction III was tested on substrate specifity. Fraction III showed three different proteolytic activities. The question is whether the three different proteolytic activities trace back to the activity of one enzyme or to the interaction of several enzymes.
Fraction III was able to convert angiotensin-I substrate to angiotensin-II (figure 28a). Therefore Fraction III had dipeptidyl-peptidase-like activity. Fraction III also had endopeptidase like activity-it was capable to convert renin tetradecapeptide substrate directly to angiotensin-II (figure 28b). No intermediate angiotensin-I was formed. Enzymes such as cathepsin G, chymase and tonin belong to the family of endopeptidase with chymotrypsin-like activity (87). Among these endopeptidases enzymes such as cathepsin G (65), tonin (36) and kallikrein (31, 37) are able to convert angiotensin-I and renin tetradecapeptide substrate directly to angiotensin-II. So therefore the angiotensin-II-generating enzyme(s) of Fraction III might belong to the same family of endopeptidases. An interaction of two proteolytic activities- one that converts renin tetradecapeptide substrate to angiotensin-I first and then one that converts angiotensin-I to angiotensin-II-can be excluded. Renin is the only enzyme known to convert renin tetradecapeptide substrate to angiotensin-I. Incubation in presence of a specific renin inhibitor did not inhibit angiotensin-II-generating activity (figure 28c).

The Low-molecular-weight-kininogen analogue Lys-Bradykinin-Ser-Val-Gln-Val-Ser-substrate for detection of kallikrein-like protease activities was cleaved by Fraction III after the phenylalanine^{9} residue of the amino acid sequence of the substrate (figure 29) to form Des-Arg^{10}-Kallidin. Hence Fraction III proved to have chymotrypsin-like activity, e.g. it hydrolyzes peptide bonds formed by the carboxyl groups of aromatic amino acids of tyrosine, tryptophan, phenylalanine and leucine. Proteases with kallikrein-like activities would have cleaved the L-kininogen analogue Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate after the arginine^{10} residue to form kallidin (molecular weight 1188 Da) (88). Such kallikrein-like activity could not be observed. Further Fraction III did not degrade kallidin (data not shown). Cathepsin G and

chymase would have hydrolyzed kallidin or bradykinin after the phenylalanine^{9} residue to form Des-Arg^{10}-Kallidin (89). Cathepsin G and Chymase are not able to hydrolyze the low-molecular-weight-kininogen analogue Lys-Bradykinin-Ser-Val-Gln-Val-Ser to form Des-Arg^{10}-Kallidin directly. Tonin is capable to cleave low-molecular-weight-kininogen directly and releases kallidin but not Des-Arg^{10}-Kallidin (90). Angiotensin converting enzyme converts angiotensin-I to angiotensin-II by cleaving the carboxy-terminal His^{9}-Leu^{10}-dipeptide. Angiotensin converting enzyme also degrades bradykinin/kallidin by cleaving the carboxy-terminal Pro^{7}-Phe^{8} bond and further the Phe^{5}-Ser^{6} bond (91). ACE is the major bradykinin/kallidin degrading enzyme. Angiotensin converting enzyme is not able to hydrolyze the L-kininogen analogue Lys-Bradykinin-Ser-Val-Gln-Val-Ser directly (92). The substrate specifity of Fraction III is a unique feature and does not coincide with the known proteases of the Kinintensin system.

The database search for a matching enzyme with the same substrate specifity using the BRENDA enzyme database and the MEROPS peptidase database yielded no entry. But the databases entries maybe considered as incomplete since enzymes such as tonin, Cathepsin G and angiotensin converting enzyme are not included with their full substrate specifity. The following table 10 gives an overview of the substrate specifity of Fraction III within the peptide metabolism in the kinintensin-system.

Substrate	**Product**	**Enzyme**	**Reaction**
renin tetradecapeptide substrate (1759 Da)	angiotensin-I (1296Da)	renin	Asp^{1} –Arg^{2} –Val^{3} –Tyr^{4}-Ile^{5}-His^{6} –Pro^{7}-Phe^{8}-His^{9}-Leu^{10}┼Leu^{11}-Val^{12}-Tyr^{13}-Ser^{14}
angiotensin-I (1296Da)	angiotensin-II (1046 Da)	ACE, Chymase, Tonin, Kallikrein, Cathepsin G, **Fraction III**	Asp^{1} –Arg^{2} –Val^{3} –Tyr^{4}-Ile^{5}-His^{6} –Pro^{7}-Phe^{8}┼His^{9}-Leu^{10}
renin tetradecapeptide substrate (1759 Da)	angiotensin-II (1046 Da)	Tonin, Kallikrein, Cathepsin G, **Fraction III**	Asp^{1} –Arg^{2} –Val^{3} –Tyr^{4}-Ile^{5}-His^{6} –Pro^{7}-Phe^{8}┼His^{9}-Leu^{10}-Leu^{11}-Val^{12}-Tyr^{13}-Ser^{14}
L-kininogen analogue: Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate (1688 Da)	Kallidin (1188 Da)	tissue kallikrein Tonin	Lys^{1} –Arg^{2} –Pro^{3} –Pro^{4}-Gly^{5}-Phe^{6} –Ser^{7}-Pro^{8}-Phe^{9}-Arg^{10}┼Ser^{11}-Val^{12}-Gln^{13}-$Val^{14}Ser^{15}$
L-kininogen analogue: Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate (1688 Da)	Des-Arg^{10}-Kallidin (1032 Da)	**Fraction III**	Lys^{1} –Arg^{2} –Pro^{3} –Pro^{4}-Gly^{5}-Phe^{6} –Ser^{7}-Pro^{8}-Phe^{9}┼Arg^{10}-Ser^{11}-Val^{12}-Gln^{13}-$Val^{14}Ser^{15}$
Kallidin (1188 Da)	Des-Phe^{9}, Arg^{10}-Kallidin (857 Da)	ACE	Lys^{1} –Arg^{2} –Pro^{3} –Pro^{4}-Gly^{5}-Phe^{6}┼Ser^{7}-Pro^{8}┼Phe^{9} -Arg^{10}
Kallidin (1188 Da)	Des-Arg^{10}-Kallidin (1032 Da)	Cathepsin G, Chymase	Lys^{1} –Arg^{2} –Pro^{3} –Pro^{4}-Gly^{5}-Phe^{6} –Ser^{7}-Pro^{8}-Phe^{9}┼Arg^{10}

Table 10 Overview over the peptide metabolism of the kinintensin-system

In this work three different angiotensin-II-generating enzyme fraction were successfully purified from porcine kidney tissue extract. One of the purified enzymes was identified as angiotensin converting enzyme. The purification of angiotensin converting enzyme is the proof of principle that the MES-assay system and the PPS-system are efficient tools enabling the fast and reliable detection and purification of target angiotensin-II-generating enzymes.

A second angiotensin-II-generating enzyme was identified as Cathepsin G. Future research should find out to whether cathepsin G plays a significant role in the in vivo angiotensin-II production in kidney. A third angiotensin-II-generating enzyme fraction was purified. The identification of this protein failed. In order to identify this protein a new attempt to purify larger quantities of this enzyme should be made. A de novo sequencing of its complete or partial amino acid sequence should reveal the identity of this angiotensin-II-generating fraction.