4. Results

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

4.1.1 Detection of angiotensin-II-generating enzyme activities

Figure 10 shows the MALDI-TOF MS spectra of the reaction products of the incubation of immobilized porcine Angiotensin converting enzyme (ACE) and porcine renal tissue protein extract (KE) with angiotensin-I. ACE shows angiotensin-II-generating activity and angiotensin-I is converted to angiotensin-II. Thus the signal intensity of the angiotensin-I substrate (m/z=1297) decreases time-dependently while the angiotensin-II product signal (m/z=1047) increases. Besides angiotensin-II-generating activity porcine renal tissue protein extract contains also an endopeptidase-like enzyme activity, angiotensin-I is converted to angiotensin (1-7) (m/z=900).

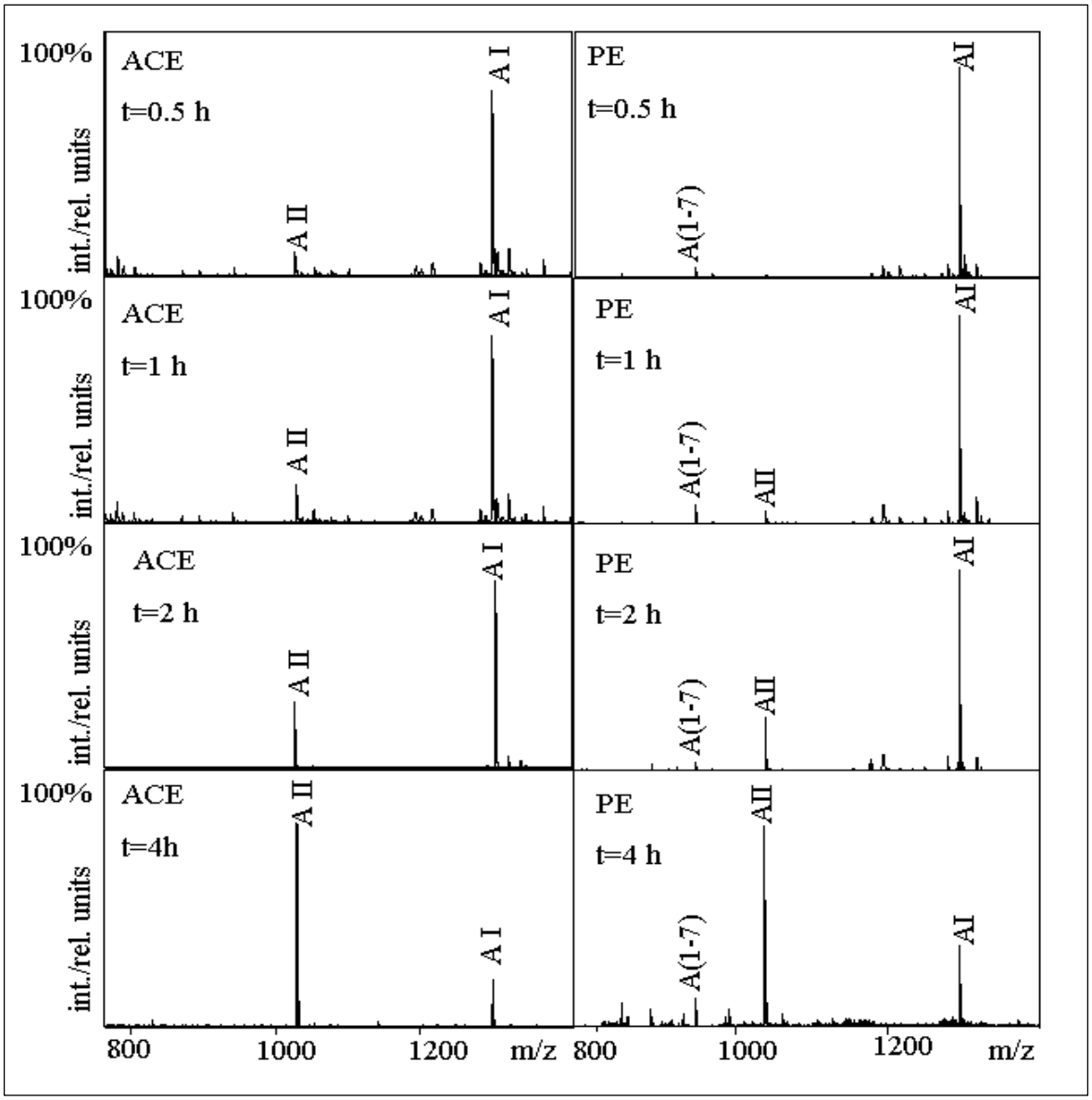


Figure 10 MALDI-MS spectra of the reaction products of the incubation of immobilized porcine ACE and porcine renal tissue protein extract (PE) with angiotensin-I substrate after different incubation times AI: Angiotensin-I (m/z=1297), A (1-7): Angiotensin (1-7) (m/z=900), AII: Angiotensin-II (m/z=1047)

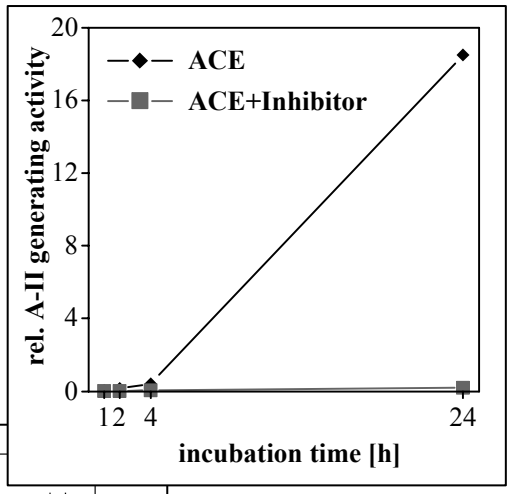
4.1.2 Correlation between enzyme activity and MES-signal intensity

Figure 11b shows the MALDI-TOF MS spectra of the reaction products of the incubation of immobilized porcine Angiotensin converting enzyme (ACE) with angiotensin-I in presence and absence of an ACE specific inactivator. ACE shows a high angiotensin-II-generating activity in absence of the inactivator and angiotensin-I is converted to angiotensin-II. Thus the signal intensity of the angiotensin-I substrate (m/z=1297) decreases time-dependently while the angiotensin-II product signal (m/z=1047) increases. After 24 h almost all of the angiotensin-I substrate is converted to angiotensin-II. The MALDI-MS spectrum shows just a small mass signal of angiotensin-I but a high signal of angiotensin-II.

ACE shows a low angiotensin-II-generating activity in presence of the ACE specific inactivator. The angiotensin-II product signal of the immobilized ACE in presence of the inactivator increases much slower compared to the angiotensin-II signal of the ACE in absence of the inactivator. Whereas at 4 h incubation time the intensity of the angiotensin-II product signal of the ACE in absence of the inactivator reaches about the half of the intensity of the angiotensin-I substrate, the angiotensin-II product signal intensity of the ACE in presence of the inactivator is less then one tenth of the intensity of the angiotensin-I substrate. After 24 h the intensity of the angiotensin-II product signal of the ACE in presence of the inactivator reaches one fifth of the intensity of the angiotensin-I substrate. A low angiotensin-II-generating enzyme activity leads to a low signal intensity of angiotensin-II measured by the MES-assay.

In figure 11a the calculated relative angiotensin-II-generating activities of the ACE in presence and absence of the specific inhibitor (measured by the MES-assay) are plotted versus the incubation time. As shown in the diagram (figure 11a) the calculated relative angiotensin-II-generating activity of the ACE in absence of the specific inactivator shows a steady rise and reaches its peak at 24 h incubation time. In contrast the calculated relative angiotensin-II-generating activity of the ACE in presence of the specific inactivator remains low and there is no significant rise during the incubation.

Figure 11a Diagram of the relative A-II generating activity of ACE in presence and absence of an ACE specific inactivator



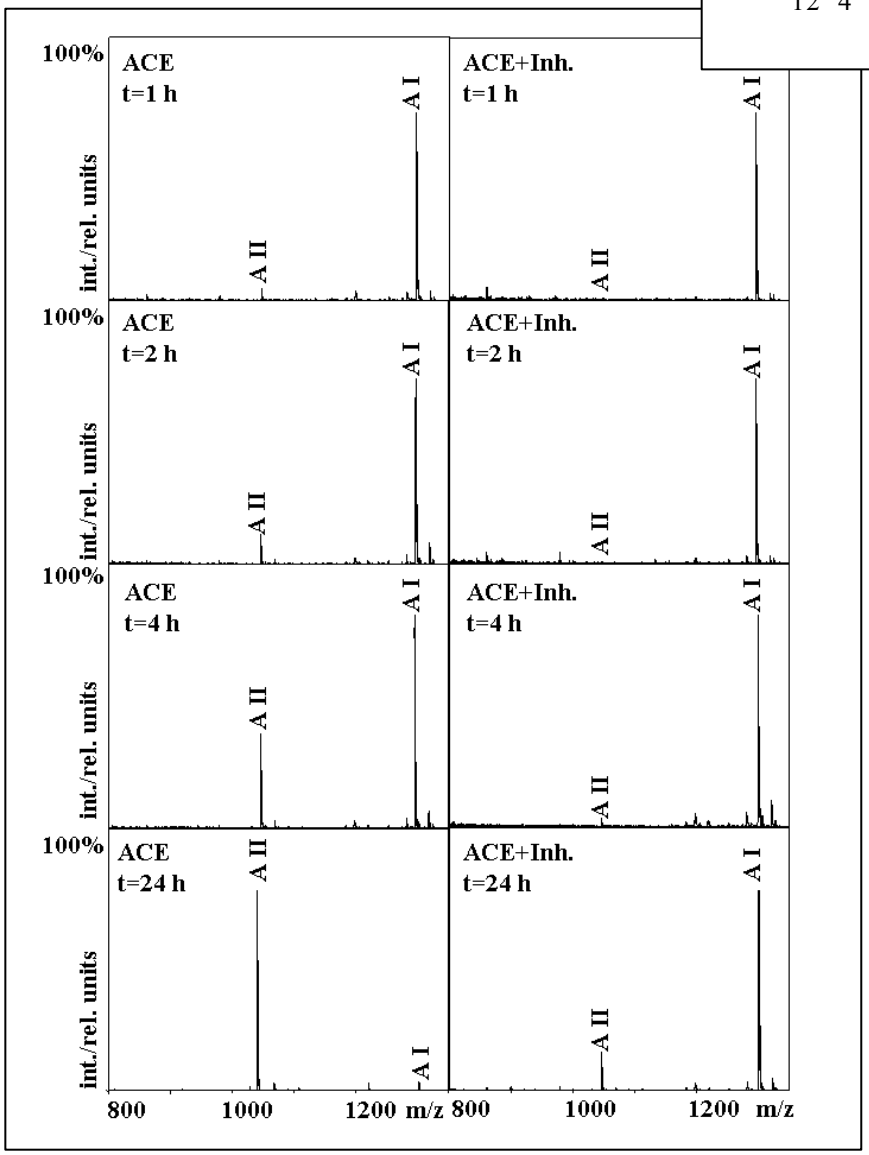


Figure 11b MALDI-MS spectra of the reaction products of the incubation of immobilized porcine ACE with angiotensin-I in presence and absence of a specific ACE inactivator after different incubation times. AI: Angiotensin-I (m/z=1297), AII: Angiotensin-II (m/z=1047)

4.1.3 Comparison of the detection sensitivity of ACE using the MES-or fluorometric enzyme assay

In figure 12a and 12b the ACE detection sensitivities of a fluorometric assay and a MALDI-MES assay are shown. The fluorometric assay system is able to detect ACE down to fmol (10^{-15} mol) amounts, the MES-assay system is even able to detect amol (10^{-18} mol) amounts of the enzyme. The MES-assay proved to be about 1000fold more sensitive than the fluorometric assay system.

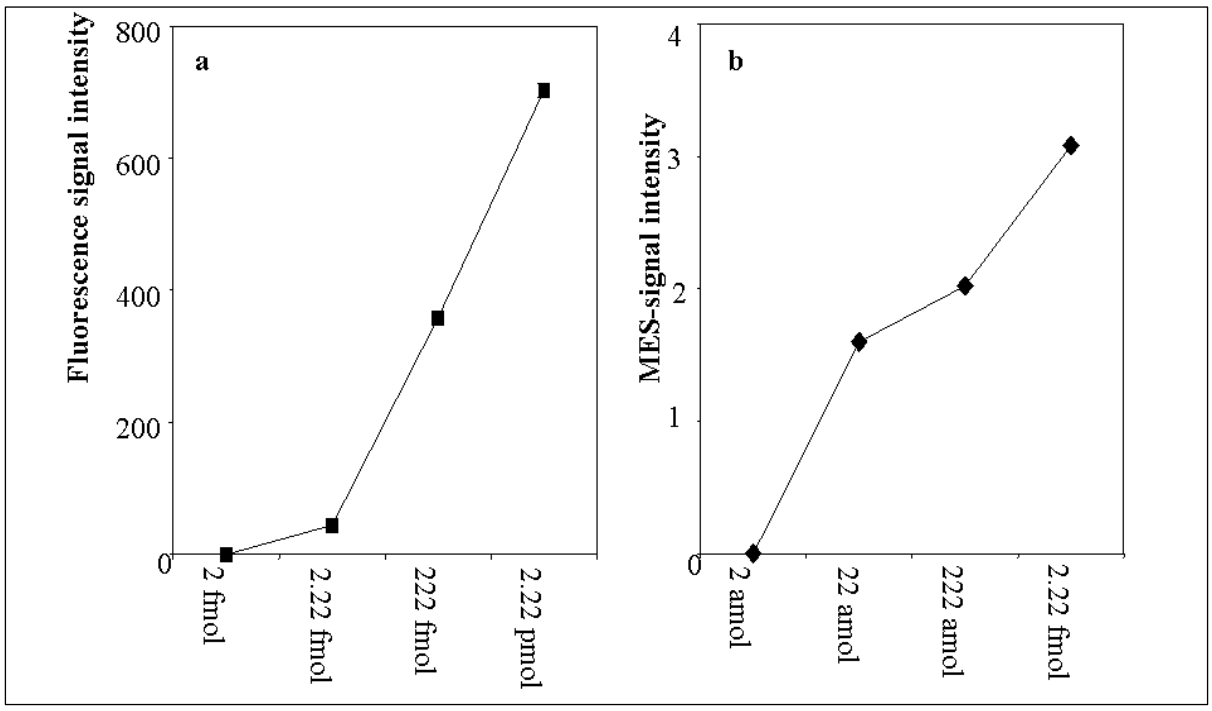


Figure 12a Fluorescence signal intensities of the reaction product of the conversion of Hip-His-Leu-OH to His-Leu-OH by different amounts of ACE **b)** MES signal intensities of the reaction product of the conversion of angiotensin-I to angiotensin-II by different amounts of immobilized ACE

4.1.4 Measurement of angiotensin-II-generating activity of renal tissue protein extract in presence of protease inhibitors

The effect of different protease inhibitors to angiotensin-II-generating enzymatic activities from crude protein extract is shown in figure 13. In the presence of the ACE-specific inactivator the A-II generating activity is reduced to about 50 %. Pefablock, a serine protease inhibitor also cut down the activity to about 70 %. EDTA a metallo protease inhibitor reduced the activity to 20 % whereas the cysteine protease specific cathepsin inhibitor showed no effect.

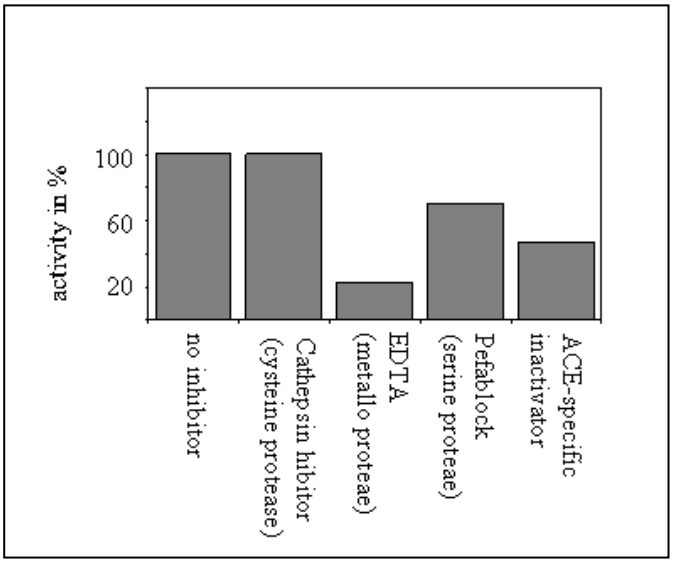


Figure 13 Maximal A-II-generating activity of an immobilized porcine renal tissue protein extract in the absence and in the presence of different protease inhibitors: metallo protease inhibitor EDTA 1mM, serine protease inhibitor Pefablock 1mM, ACE-specific inactivator 10µM, Cathepsin inhibitor 10µM.
100 % activity: activity in the absence of inhibitors.

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

4.2.1 Protein purification scouting (PPS) experiments for the determination of parameters for the purification of angiotensin-II-generating enzymes by anion exchange chromatography

The application of porcine renal tissue extract to the anion exchange protein purification scouting system yielded eluates with different levels of angiotensin-II-generating activities. As shown in table 3 the highest angiotensin-II-generating activity was found in the eluate of the batch anion exchange chromatography fraction with 0 mM NaCl and pH 9 in the equilibration buffer. Therefore for the initial purification step of porcine renal tissue an anion exchange chromatography with 0 mM NaCl and pH 9 in the equilibration buffer was used.

NaCl				**pH**
0 mM	**100 mM**	**200 mM**	**500 mM**	
0.41 (35.7%)	0.44 (5.1%)	0.41 (3.9%)	0.86 (1.5%)	**5**
1.53 (18.9%)	1.36 (13.2%)	1.58 (6.0%)	3.28 (0.6%)	**6**
4.00 (25.2%)	3.09 (13.8%)	2.38 (5.4%)	0.72 (0.3%)	**7**
3.81 (35.1%)	4.60 (20.7%)	1.20 (9.6%)	1.66 (0.6%)	**7.5**
1.26 (34.8%)	4.61 (22.8%)	1.04 (8.1%)	2.96 (0.9%)	**8**
1.99 (33.3%)	0.51 (20.7%)	1.37 (8.4%)	0.72 (0.03%)	**8.5**
4.88 **(29.4%)**	1.56 (22.7%)	0.02 (8.7%)	1.19 (0.3%)	**9**
4.21 (29.4%)	0.74 (23.1%)	0.2 (9.6%)	0.46 (0.6%)	**10**

Table 3 Maximal A-II-generating activities and protein yields (in brakes) of the eluates from the PPS experiment for the determination of optimal parameter for the purification of angiotensin-II-generating enzymes by anion exchange chromatography

4.2.2 Anion exchange chromatography of porcine renal tissue protein extract

As shown in figure 14 anion exchange chromatography of porcine renal tissue extract yielded a set of fractions with angiotensin-II-generating activities. Fraction 5-13 had angiotensin-II-generating activities. The fractions 6-11 were characterized further in the inhibitor profiling experiment.

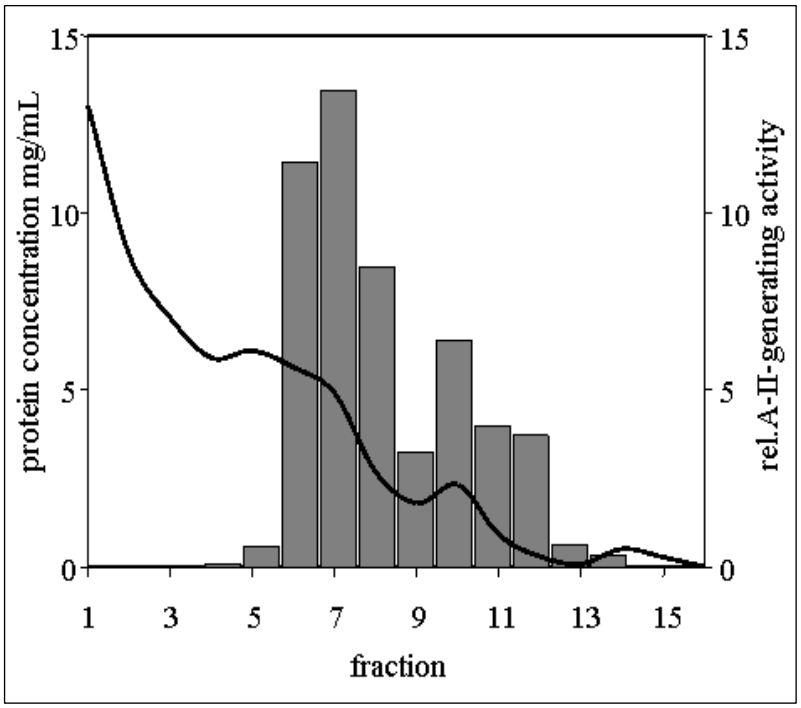


Figure 14 Chromatogram of the anion exchange chromatography of porcine renal tissue extract. The solid line represents the protein concentration, the bars the maximal relative angiotensin-II-generating activities. Protein concentration was measured using the Bradford method. Gel: Fractogel EMD TMAE (Merck), Equilibration buffer: 50 mM AMPSO pH 9, elution buffer: 50 mM AMPSO, 2M NaCl pH 9 Fraction size: 60 mL total volume

4.2.3 Inhibitor profiling of the eluates of the anion exchange chromatography with angiotensin-II-generating activities

Antipain and chymostatin are reversible inhibitors of serine and some cysteine proteases. As shown in figure 15 the eluted protein fractions were inhibited in their A-II-generating activity by antipain and/or chymostatin in a varying extent. The protein fractions 6-8 showed a strong inhibition of their angiotensin-II-generating activities in presence of antipain and chymostatin, fraction 9 just in presence of chymostatin. The fraction 10-11 showed very little or no inhibition of their angiotensin-II-generating activities in presence of antipain and chymostatin.

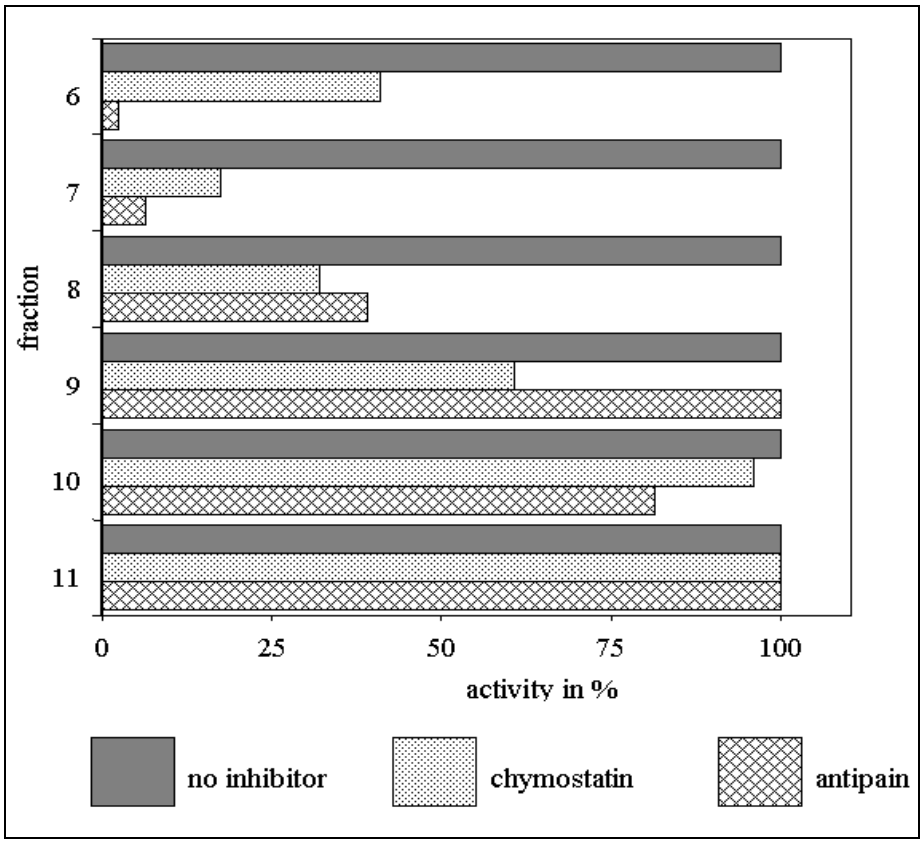


Figure 15 Angiotensin-II-generating activities of the anion exchange fraction 6-11 in presence and absence of the protease inhibitors antipain and chymostatin (inhibitors of serine and cysteine proteases), 100% activity: activity in absence of inhibitors

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

The application of the pooled aliquot of the eluates of fraction 6-9 of the anion exchange chromatography (figure 14) to a protein purification scouting experiment yielded eluates with different levels of angiotensin-II-generating activities. As shown in table 4 the highest angiotensin-II-generating activity was detected in the eluate of the hydroxyapatite chromatography. Therefore for the intermediate chromatographic purification step hydroxyapatite chromatography was chosen.

Chromatography	Maximal A-II-generating activity	protein yield [%]
Methyl-HIC	1.10	12.57
t-Butyl-HIC	0	5.71
Propyl-HIC	0.27	6.29
Ether-HIC	1.00	6.86
Hexyl-HIC	2.10	11.43
Butyl-Sepharose FF-HIC	0.63	12.00
Isopropyl-HIC	0.4	10.29
Octyl-HIC	0.97	13.71
Phenyl-HIC	1.0	18.86
Ceramic Hydroxyapatite	**16.33**	**11.66**

Table 4 Maximal A-II-generating activities and protein yields of eluates from the PPS experiment for the determination of optimal parameter for the intermediate chromatographic purification step

4.2.5 Hydroxyapatite chromatography

The angiotensin-II-generating fractions 6-9 of the anion exchange chromatography (figure 14) were pooled and applied to a hydroxyapatite chromatography. As shown in figure 16, hydroxyapatite chromatography yielded three fractions (number 1-3) with angiotensin-II-generating enzyme activity. These fractions were pooled and further purified with a lentil lectin affinity chromatography.

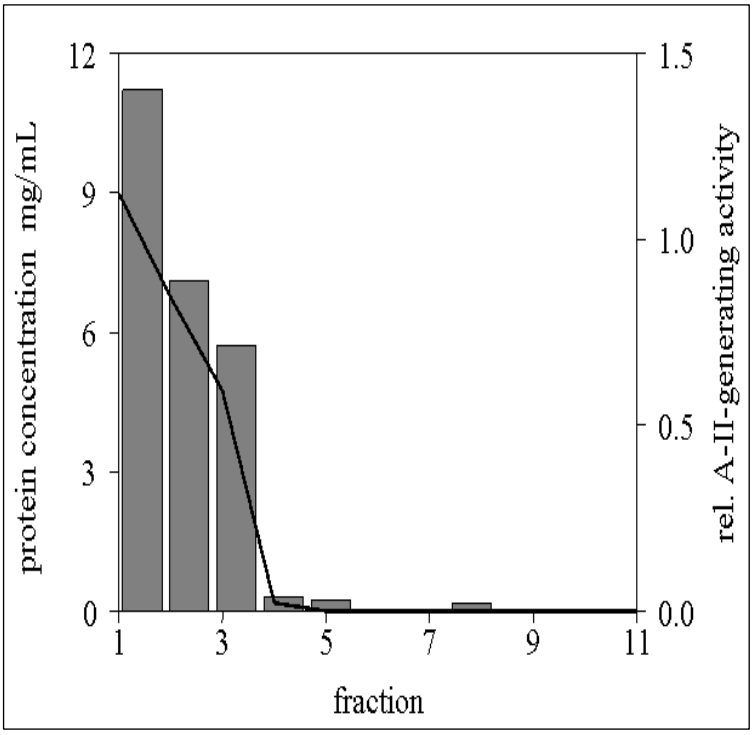


Figure 16 Chromatogram of the hydroxyl apatite chromatography of the angiotensin-II-generating fractions 6-9 of anion exchange chromatography (figure 14). The solid line represents the protein concentration, the bars the maximum of the angiotensin-II-generating activities. Protein concentration was measured using the Bradford method. Gel: CHT ™ ceramic hydroxyapatite Bio-Gel (Biorad), Equilibration buffer: 10 mM potassium phosphate pH 7, Elution buffer: 500 mM potassium phosphate pH 7, fraction size: 30 mL total volume

4.2.6 Protein purification scouting (PPS) experiments for the determination of parameters of lentil lectin affinity chromatography

Aliquots of the pooled fractions 1-3 from hydroxyapatite chromatography (figure 16) were applied to the PPS experiment using lentil lectin sugar affinity chromatography. Within this PPS experiment the composition of the elution buffer was varied. As shown in table 5 the highest angiotensin-II-generating activity was found in the protein fraction that eluted by the elution buffer 1, which contained 250 mM Methyl-α-D-glycopyranoside.

Elution buffer	Maximal A-II-generating activity	Protein yield [in % of applied initial sample amount]
1	**1.34**	**8.0**
2	0.312	10.0

Table 5 Maximal A-II-generating activities and protein yields of the eluates from the PPS experiment for the determination of optimal parameter of lentil lectin chromatography, Elution buffer 1: 20 mM TRIS-HCl, 250 mM Methyl-α-D-glycopyranoside, pH 7.4.
Elution buffer 2: 20 mM TRIS-HCl, 250mM Methyl-α-D-mannopyranoside, pH 7.4

4.2.7 Lentil Lectin affinity chromatography

Figure 17 shows the chromatogram of the Lentil Lectin affinity chromatography of the angiotensin-II-generating fraction 1-3 form hydroxyapatite chromatography (figure 16). The lentil lectin binds α-D-glucose and α-D-mannose residues of glycoproteins. The lentil lectin affinity chromatography yielded two different angiotensin-II-generating protein fractions. The first fraction did only bind weakly to the lectin ligands and was washed out before elution (Fraction A). The second fraction did bind strongly to the lectin ligand and was eluted by using a gradient of the competitive sugar 250 mM Methyl-α-D-glycopyranoside (Fraction B). For further characterization of the enzymatic activities both angiotensin-II-generating activities were subjected to an inhibitor profiling experiment.

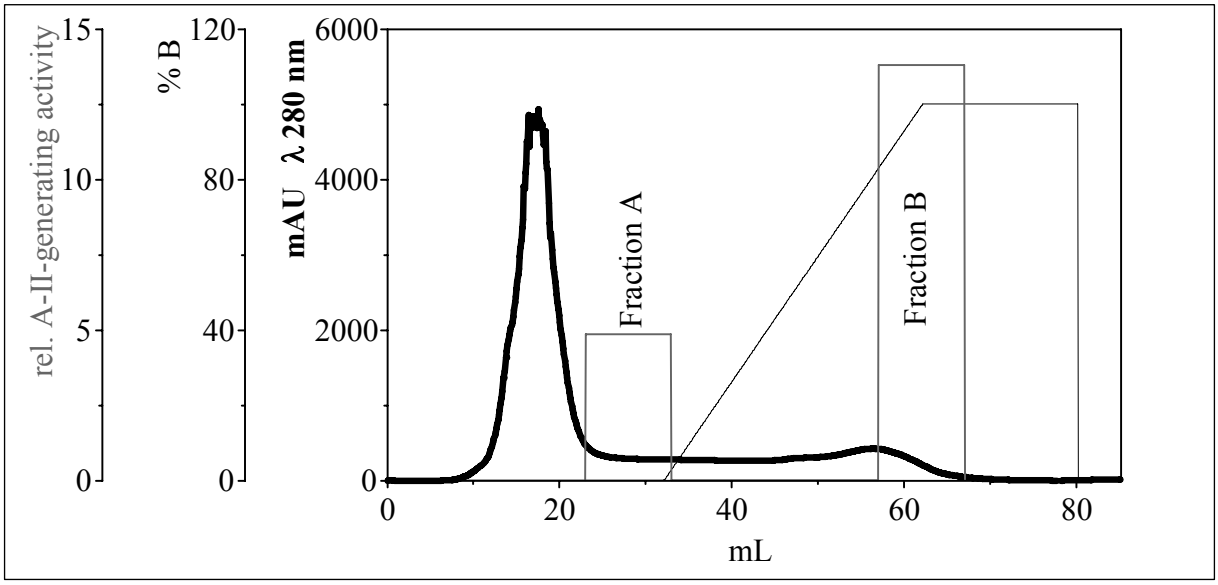


Figure 17 Chromatogram of the lentil lectin affinity chromatography of the angiotensin-II-generating fraction 1-3 form hydroxyapatite chromatography (figure 16). The solid fat line represents the protein concentration, the solid fine line the concentration of elution buffer B, the bars the maxima of the A-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Gel: lentil lectin sepharose 4B (Amersham Biosciences) Equilibration buffer: 20 mM Tris-HCl, 500 mM NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4, elution buffer: 20 mm TRIS-HCl, 250 mM Methyl-α-D-glycopyranoside, pH 7.4, fraction volume: 10 mL total volume

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

The Fraction A of the lentil lectin chromatography (figure 17) showed inhibition of A-II-generating activity in presence of chymostatin and antipain (figure 18). Thus the enzyme which is responsible for this activity must be probably of serine or cysteine like proteases type.
Fraction B of the lentil lectin chromatography (figure 17) showed an inhibition of A-II-generating activity in presence of EDTA, an inhibitor of metallo proteases, and in presence of the ACE-specific inactivator. Thus the enzyme which is responsible for this activity must be a metallo protease and must have a structural similarity to the angiotensin converting enzyme. Fraction A which showed inhibition of the enzymatic activity in presence of antipain and chymostatin was applied to an inhibitor affinity chromatography with antipain and chymostatin as ligand. Fraction B which showed inhibition in presence of EDTA and ACE-specific inactivator was further purified using size exclusion chromatography.

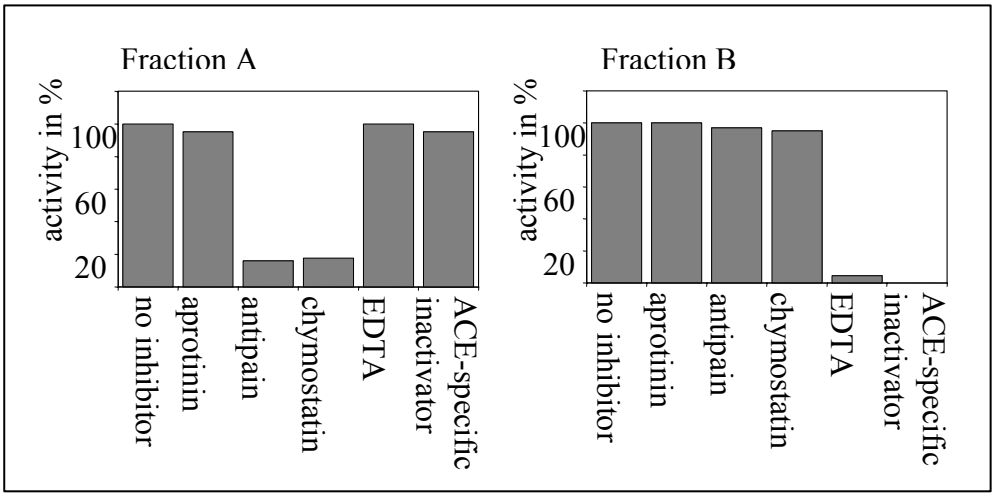


Figure 18 Maximal A-II-generating activity of the immobilized eluates from the weakly binding fraction (Fraction I) and binding fraction (Fraction II) of the lectin affinity chromatography (figure 17) in the absence and in the presence of different protease inhibitors: antipain (inhibitor of serine and cysteine proteases), chymostatin (inhibitor of serine and cysteine proteases), EDTA (metallo protease inhibitor), ACE-specific inactivator, aprotinin (serine protease inhibitor), 100 % activity: activity in the absence of inhibitors

4.2.9 Chymostatin-Antipain affinity chromatography

The Fraction A of the lentil lectin chromatography (figure 17) was applied to the chymostatin-antipain-inhibitor affinity chromatography. One protein fraction with angiotensin-II-generating activity was eluted from the chromatography gel with a pH gradient as shown in figure 19. This fraction was named Fraction I and passed to the protein identification experiments.

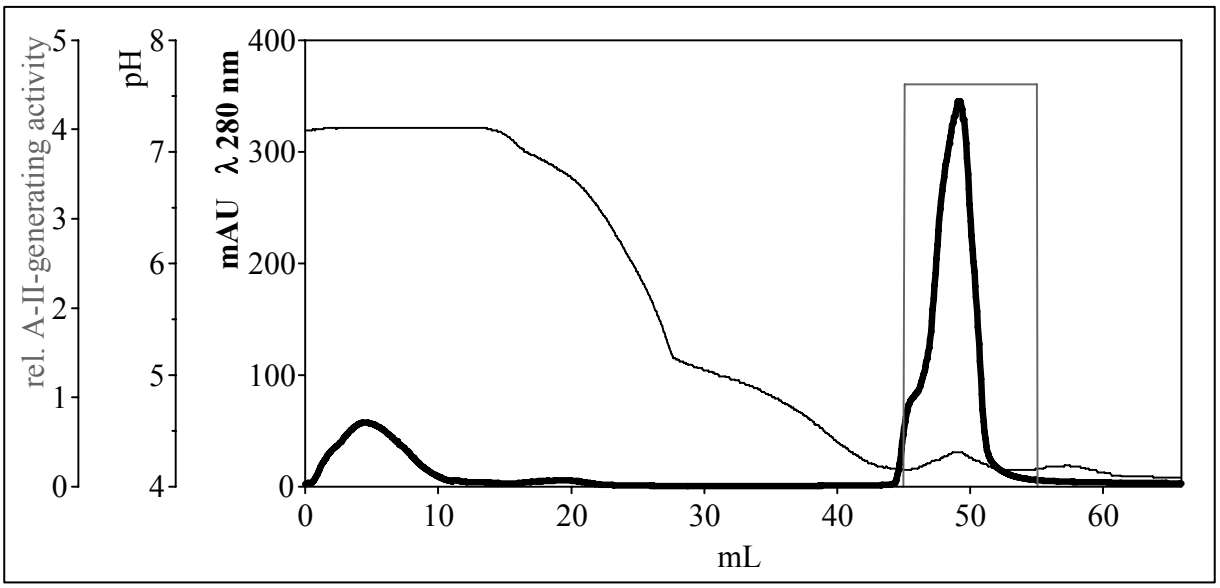


Figure 19 Chromatogram of the chymostatin-antipain-inhibitor affinity chromatography of the angiotensin-II-generating Fraction A of the lentil lectin chromatography (figure 17). The solid fat line represents the protein concentration, the solid fine line the pH of elution buffer B, the bar the maximum of the angiotensin-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Gel: antipain/chymostatin (ligand density 1 mM) on EAH-Sepharose 4B (Amersham Biosciences) Equilibration buffer: 50 mM HEPES, 500 mM NaCl, pH 8 elution buffer: 50 mM potassium phosphate pH 4. Fraction size: 10 mL total volume

4.2.10 Size exclusion chromatography of Fraction B of lentil lectin chromatography

Previous to the chromatography of the sample (Fraction B of lentil lectin chromatography) a chromatography of a mixture of standard proteins and peptides was done in order to prepare a calibration curve (figure 21) of the partition coefficient K_{av} versus the molecular weights of the standard proteins and peptides.

Table 6 shows the elution volumes and the calculated partition coefficients. The void volume was determined using blue dextran and was 7.7 mL. The total volume of the column was 24 mL. Using size exclusion chromatography one angiotensin-II-generating fraction was found with an elution volume of 12 to 13 mL as shown in the chromatogram (figure 20). The calculated partition coefficient K_{av} was 0.26-0.29. The molecular weight of that protein was approximated with the help of the calibration curve (figure 21). This angiotensin-II-generating protein in the fraction had a molecular weight of approximately 200 kDa. This fraction was named Fraction II and passed to the protein identification experiments.

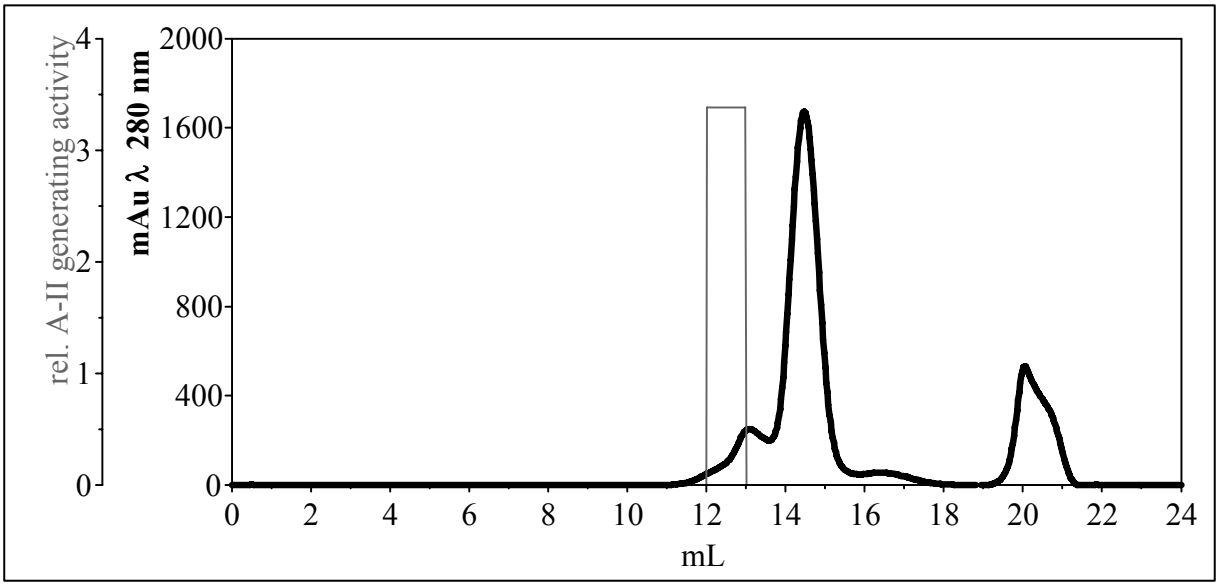


Figure 20 Chromatogram of the size exclusion chromatography of the angiotensin-II-generating fraction B of the lentil lectin chromatography (figure 17). The solid fat line represents the protein concentration, the bar the maximum of the angiotensin-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Column: Superdex 200 HR 10/30 (Amersham Biosciences), running buffer: 50 mM potassium phosphate, 150 mM NaCl, pH 7. Fraction size: 1 mL total volume

	Molecular weight (kDa)	Elution volume (mL)	calculated partition coefficient K_{av}
Thyroglobulin	669	9.60	0.11
Ferritin	440	11.06	0.21
Immunoglobulin G	160	12.50	0.29
Bovine serum albumin	67	14.20	0.4
β-Lactoglobulin	35	15.50	0.48
cytochrome C	12.4	17.30	0.59
Vitamin B12	1.4	20.00	0.75
Angiotensin-II-generating Fraction II of the lentil lectin chromatography	*approximately 200*	*12.00-13.00*	*0.26-0.29*

Table 6 Elution volume and calculated partition coefficients (K_{av}) of standard molecules and the sample Fraction II (italics**)**

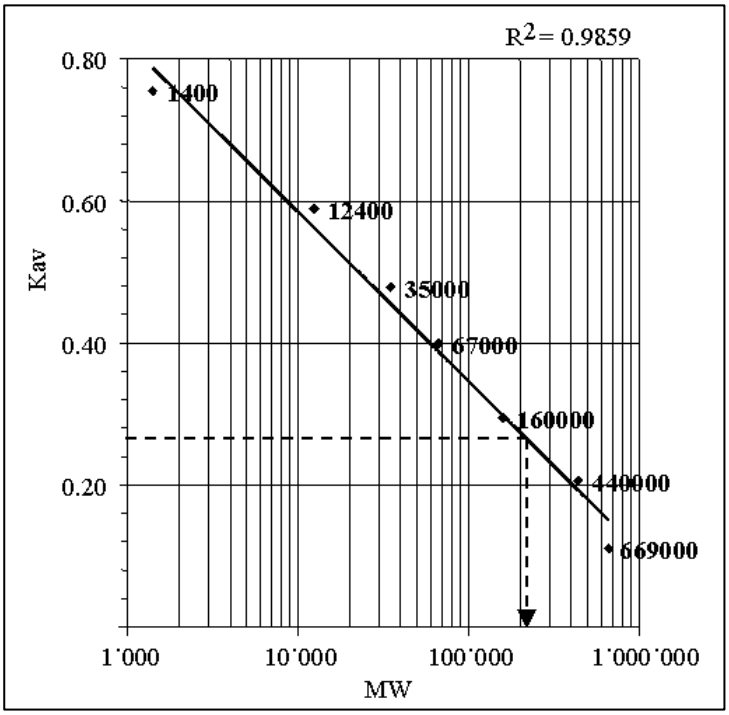


Figure 21 Calibration curve of the size exclusion chromatography experiment (figure 20).
X-axis plotted in logarithmic scale.
Dotted line with arrow: calculated partition coefficient of the angiotensin-II-generating fraction II, MW: molecular weight in Da

4.3 Part III- Purification of an angiotensin-II-generating enzyme from porcine renal tissue protein extract

4.3.1 Anion exchange chromatography of porcine renal tissue protein extract

As shown in figure 22 anion exchange chromatography of porcine renal tissue extract yielded one eluate fraction with angiotensin-II-generating activity. This fraction eluted with the increasing salt gradient of elution buffer. This fraction was applied to wheat germ lectin chromatography.

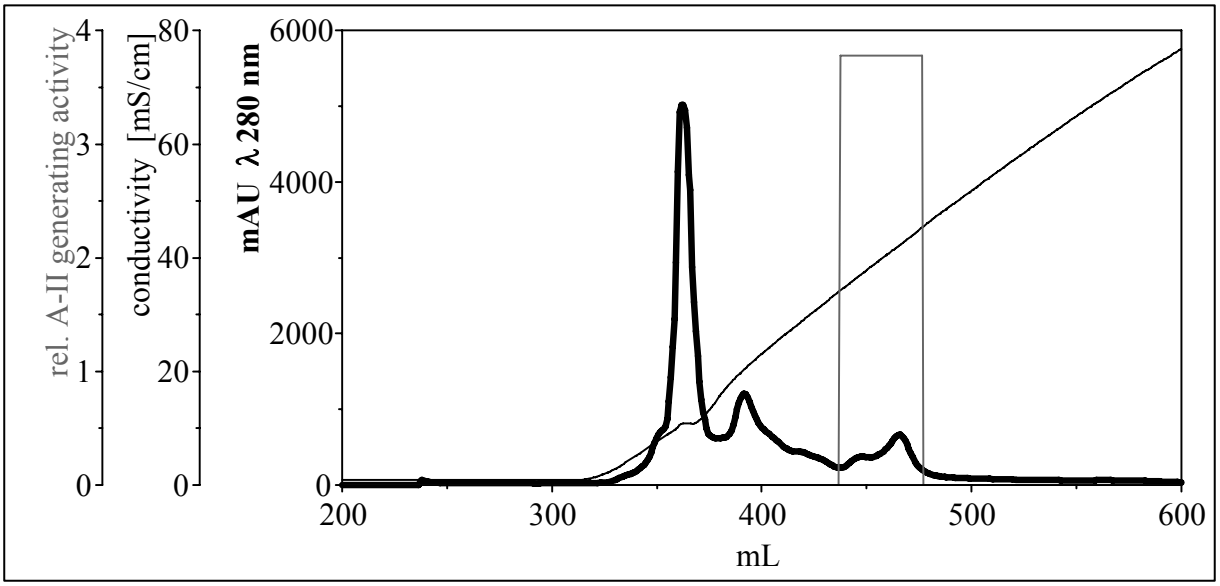


Figure 22 Chromatogram of the anion exchange chromatography of porcine renal tissue extract. The solid fat line represents the protein concentration, the solid fine line the conductivity, the bar the maximum of the angiotensin-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Gel: Fractogel™ EMD DEAE 650 M (Merck), equilibration buffer: 50 mM AMPSO, 1 mM EDTA, pH8.3, elution buffer: 50 mM AMPSO, 1 mM EDTA, 1 M NaCl, pH 8.3, fraction size: 40 mL total volume

4.3.2 Wheat germ lectin chromatography

Figure 23 shows the chromatogram of the wheat germ lectin affinity chromatography of the angiotensin-II-generating fraction from the anion exchange chromatography (figure 22). The wheat germ lectin binds the chitobiose core of N-linked (via N-acetylglucosamine) oligosaccharides. The wheat germ lectin affinity chromatography yielded one angiotensin-II-generating protein fractions. The angiotensin-II-generating enzymes did bind strongly to the

lectin ligand and were eluted by using a step elution with the competitive sugar N-acetylglucosamine.

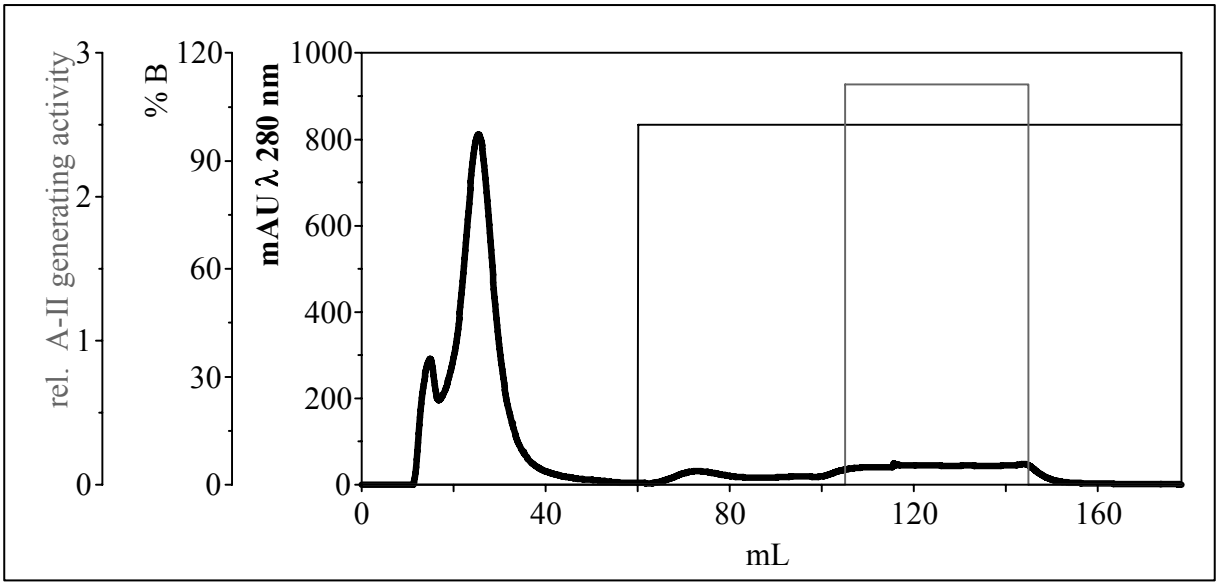


Figure 23 Chromatogram of wheat germ lectin affinity chromatography of the angiotensin-II-generating fraction from the anion exchange chromatography (figure 22). The solid fat line represents the protein concentration, the solid fine line the concentration of elution buffer B, the bar the maximum of the angiotensin-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Gel: Wheat germ lectin sepharose 4B (Amersham Biosciences) Equilibration buffer: 20 mM TRIS-HCl, 500 mM NaCl, pH 7.4, elution buffer: 20 mM TRIS-HCl, 300 mM N-acetyl-glucosamine pH 7.4, fraction size: 10 mL total volume

4.3.3 Inhibitor profiling experiment of the eluate with angiotensin-II- generating activity

There was no inhibition of angiotensin-II-generating activity of the eluate detectable in presence of the inhibitors antipain and chymostatin (figure 24). In presence of aprotinin the angiotensin-II-generating activity was reduced to 20% of the activity of the non treated aliquot. The angiotensin-II-generating fraction of wheat germ lectin was subjected to aprotinin affinity chromatography.

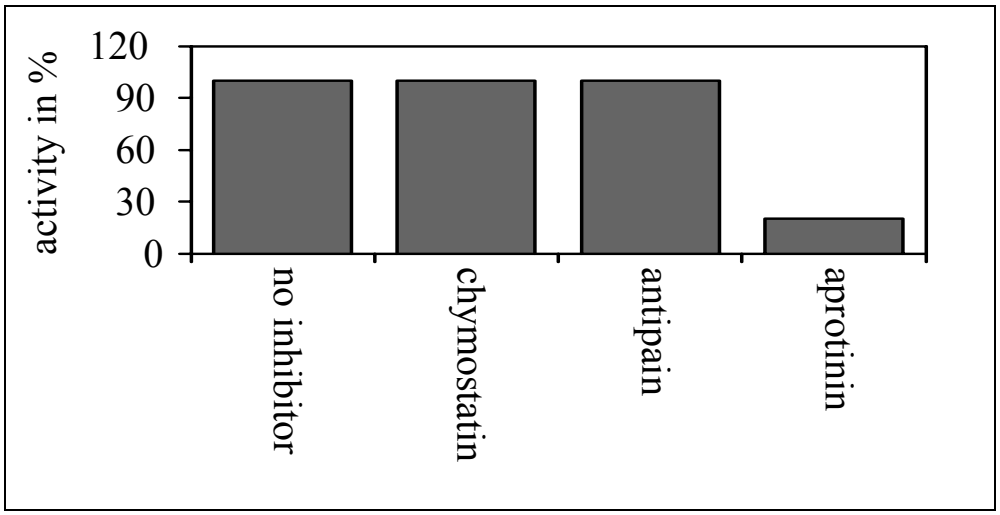


Figure 24 Angiotensin-II-generating activities of the anion exchange fraction 6-11 (figure 23) in presence and absence of the protease inhibitors antipain and chymostatin (inhibitors of serine and cysteine proteases), aprotinin (inhibitor of serine proteases, 100% activity: activity in absence of inhibitors

4.3.4 Aprotinin affinity chromatography

One protein fraction with angiotensin-II-generating activity was eluted from the chromatography gel with an isocratic pH gradient (figure 25). This fraction was named Fraction III and passed to the protein identification experiments.

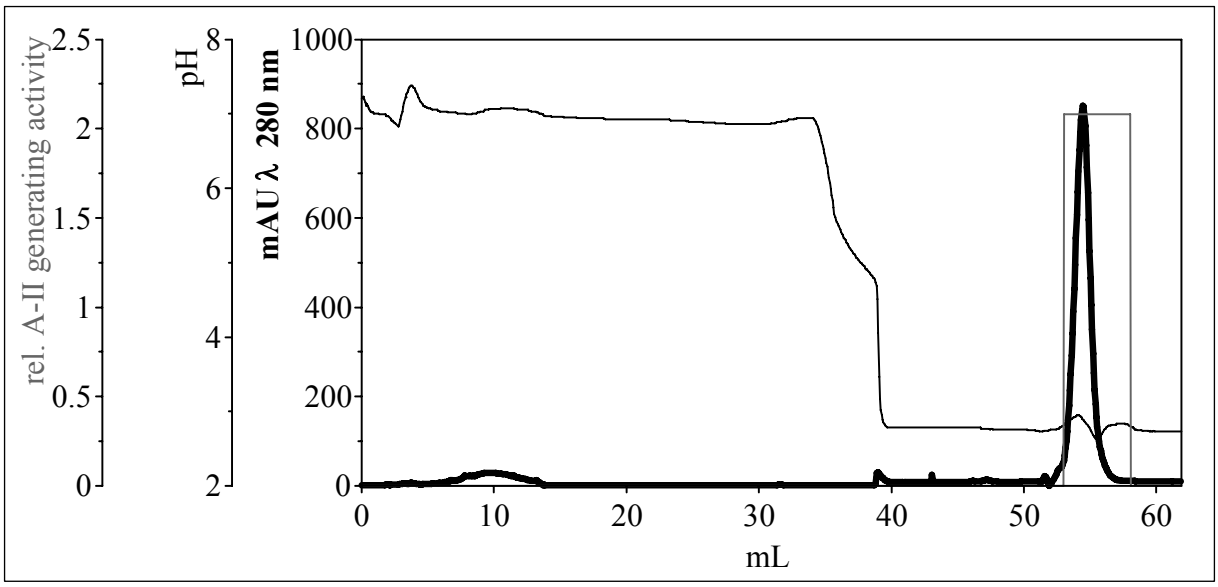


Figure 25 Chromatogram of the aprotinin- inhibitor affinity chromatography of the angiotensin-II-generating fraction from the wheat germ lectin chromatography (figure 23). The solid fat line represents the protein concentration, the solid fine line the pH of elution buffer B, the bars the maximum of the angiotensin-II-generating activity. Protein concentration was monitored at λ 280 nm UV. A-II-generating fraction was found by pH elution at pH 3.
Gel: aprotinin (ligand density 4mg/mL) on CNBr-activated -Sepharose 4B (Amersham Biosciences) Equilibration buffer: 50 mM HEPES, 500 mM NaCl, pH 8 elution buffer: 50 mM potassium phosphate pH 3. Fraction size: 5 mL total volume

4.3.5 Determination of the molecular weight of the Fraction III

Table 7 shows the determined elution volumes and the calculated partition coefficients of standard molecules and the sample Fraction III. The void volume was determined using blue dextran and was 7.7 mL. The total volume of the column was 24 mL. Using size exclusion chromatography one angiotensin-II-generating fraction was found with an elution volume of 12.5 mL as shown in the chromatogram (figure 26). The calculated partition coefficient K_{av} was 0.29. The molecular weight of that fraction was approximated with the help of the calibration curve (figure 27). This angiotensin-II-generating protein fraction had a molecular weight of approximately 160 kDa.

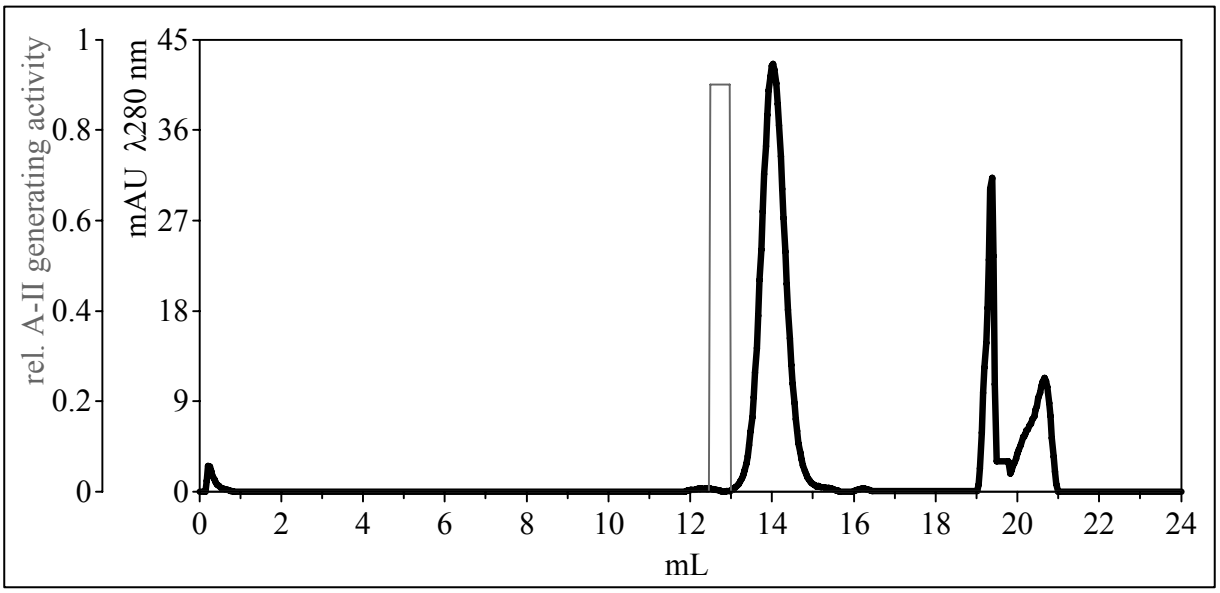


Figure 26 Chromatogram of the size exclusion chromatography of Fraction III. The solid fat line represents the protein concentration, the bars the maximum of the angiotensin-II-generating activities. Protein concentration was monitored at λ 280 nm UV.
Column: Superdex 200 HR 10/30 (Amersham Biosciences), running buffer: 50 mM potassium phosphate, 150 mM NaCl, pH 7. Fraction size 0.5 mL

	Molecular weight (kDa)	Elution volume (mL)	calculated partition coefficient K_{av}
Ferritin	440	11.06	0.21
Immunoglobulin G	160	12.50	0.29
Bovine serum albumin	67	14.20	0.40
β-Lactoglobulin	35	15.50	0.48
cytochrome C	12.4	17.30	0.59
Angiotensin-II-generating Fraction III	*approximately 160*	*12.50*	*0.29*

Table 7 Determined elution volumes and calculated partition coefficients (K_{av}) of standard molecules and the sample Fraction III (italics)

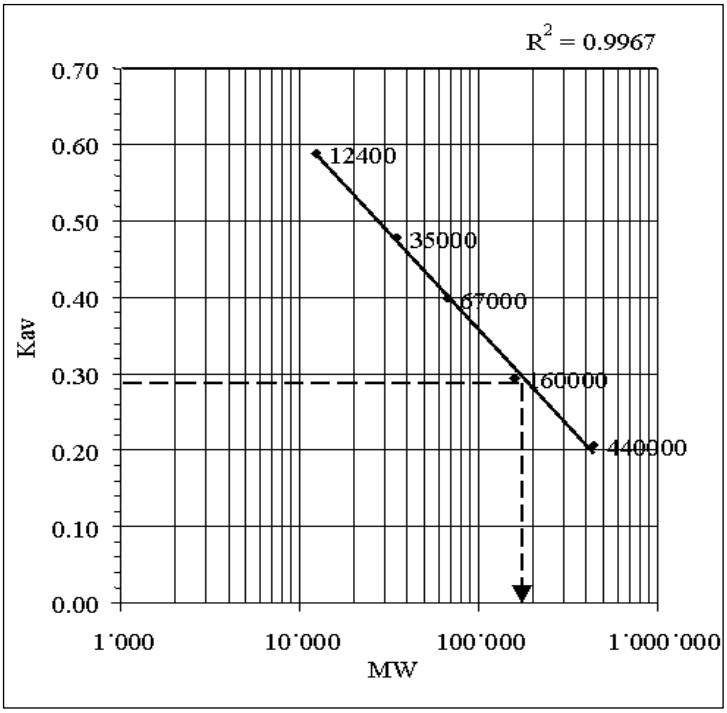


Figure 27 Calibration curve of the size exclusion chromatography experiment of the Fraction III (figure 26). X-axis plotted in logarithmic scale. Dotted line with arrow: calculated partition coefficient of the angiotensin-II-generating Fraction III

4.3.6 Test of substrate specifity of Fraction III

As shown in figure 28a Fraction III was able to cleave angiotensin-I (m/z=1297) to form the reaction product angiotensin-II (m/z=1047). It also converted the renin tetradecapeptide substrate (m/z=1760) directly to angiotensin-II (figure 28b). Therefore no intermediate angiotensin-I signal (m/z=1297) was detectable.

In presence of the renin specific inhibitor the renin tetradecapeptide substrate was converted to angiotensin-II. No angiotensin-I signal was detectable and there was no inhibition of the angiotensin-II-generating activity in presence of the renin-specific inhibitor (figure 28c). Thus the conversion of renin tetradecapeptide substrate to angiotensin-II occurred without the intermediate angiotensin-I stage.

Fraction III cleaved the L-kininogen analogue Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate (m/z=1688) between the phenylalanine^{9}-and the arginine ^{10} -residue of the amino acid sequence of the substrate (figure 29). The resulting proteolytic product is Des-Arg^{10}-Kallidin (m/z=1032). Fraction III did not degrade kallidin (data not shown).

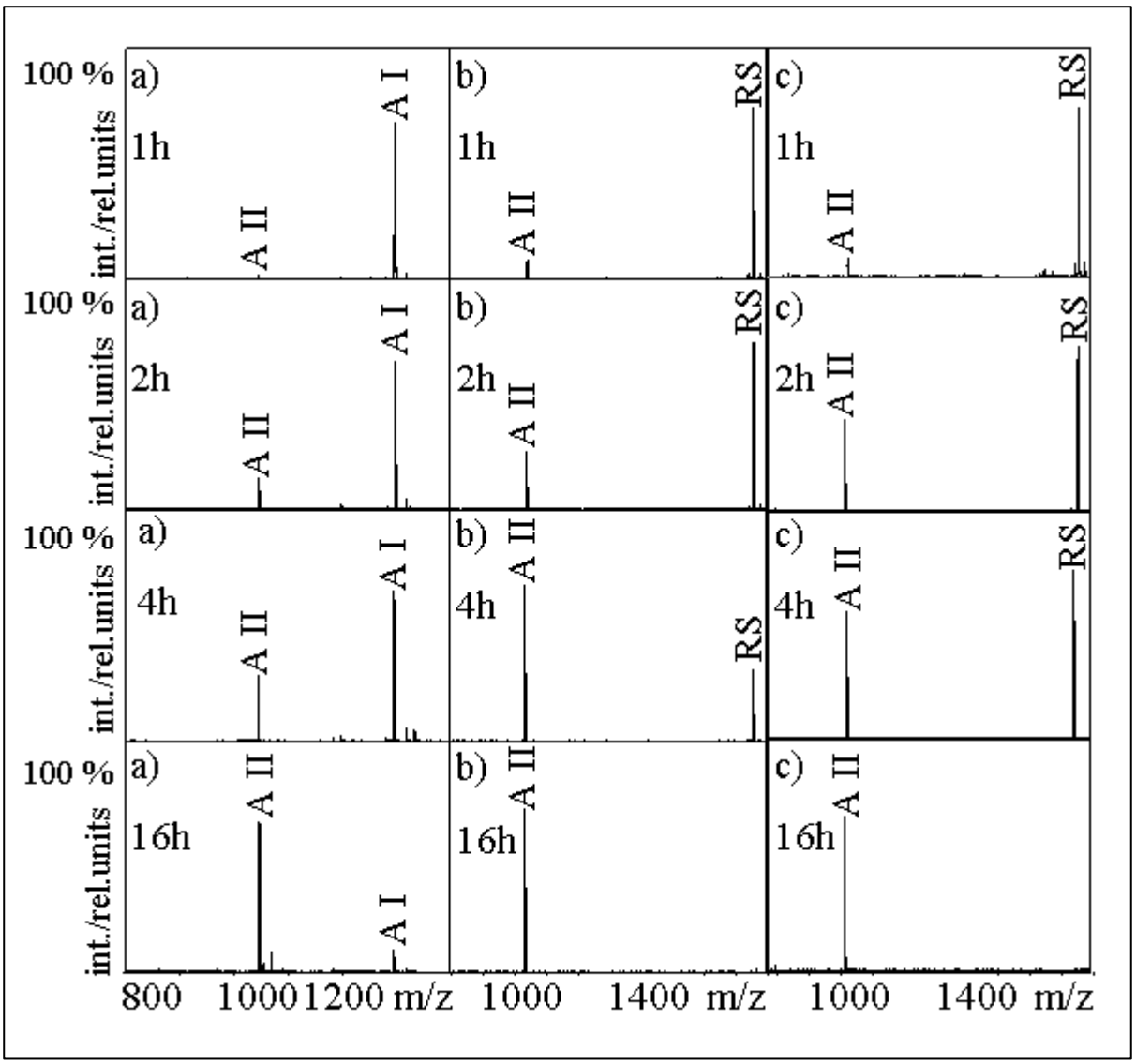


Figure 28 MALDI-MS spectra of the reaction products of the incubation of immobilized proteins of Fraction III (figure 25) with **a)** angiotensin-I, **b)** porcine renin tetradecapeptide substrate, **c)** porcine renin tetradecapeptide substrate + renin specific inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe after different incubation times.
AI: Angiotensin-I (m/z=1297), AII: Angiotensin-II (m/z=1047), RS: porcine renin tetradecapeptide substrate (m/z=1760)

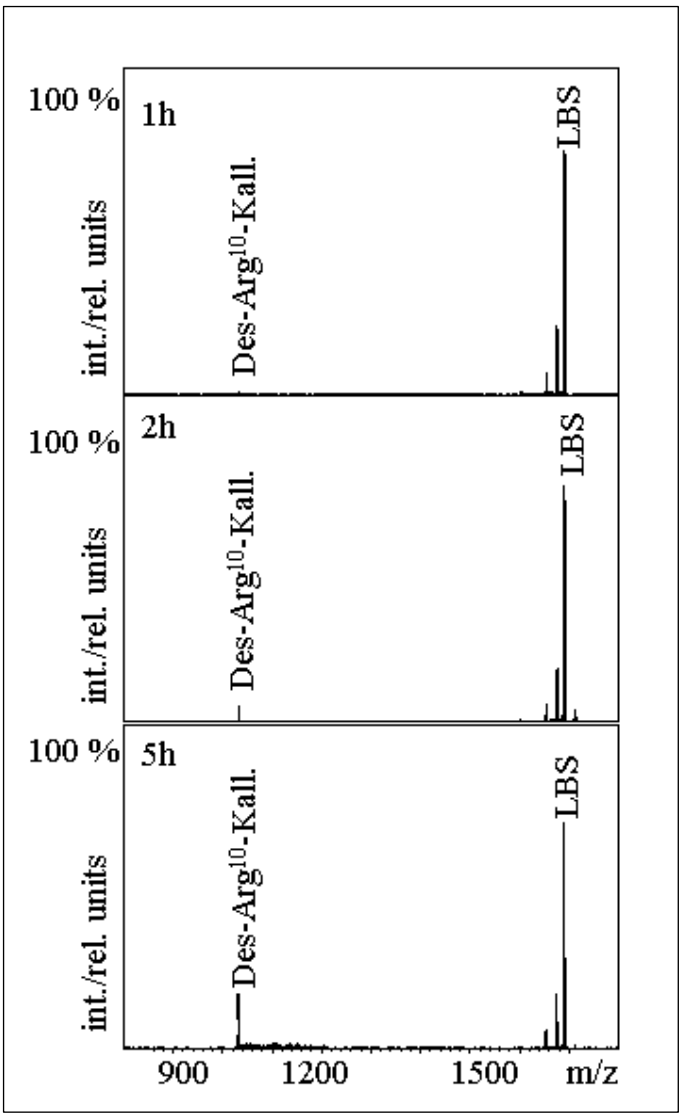


Figure 29 MALDI-MS spectra of the reaction products of the incubation of immobilized proteins of Fraction III (figure 25) with Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate after different incubation times.

LBS: Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate (m/z=1688), Des-Arg^{10}-Kall.: Des-Arg^{10}-Kallidin (m/z=1032).

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

The database matching for "angiotensin-II" using the BRENDA enzyme database and a full text search for the sub item "natural product" retrieved three entries: first peptidyl-dipeptidase A (synonymous for angiotensin converting enzyme, EC 3.4.15.1) from pig, second the same enzyme peptidyl-dipeptidase A from clam worm and third chymase from human. The database matching for "desArg^{10}-kallidin" using the BRENDA enzyme database and a full text search for the sub item "natural product" no entries whereas the more general search term "kallidin" returned one enzyme candidate, renal tissue kallikrein from human (EC 3.4.21.35). However kallidin has not the same structure as the desArg^{10}-kallidin molecule. Therefore the search result renal tissue kallikrein from human was rejected. There was no entry in the BRENDA database for an enzyme which generates both: angiotensin-II as well as the desArg^{10}-kallidin molecule.
The search using the options "search by specifity" and "How may this substrate be cleaved?" of the Merops peptidase database resulted no matching entry for the substrates angiotensin-I and renin tetradecapeptide substrate, i.e. there was no enzyme candidate included in the database that was capable to generate angiotensin-II form angiotensin-I as well as from renin substrate decapeptide. Using the option "What peptidase can cleave this bond?" in the "searches service" of the Merops database and entering the amino acid sequence of the cleavage site of the substrates angiotensin-II and renin substrate decapeptide yielded one entry: the enzyme tonin (EC 3.4.21.35). The entry of the amino acid sequence of the cleavage site of the Lys-Bradykinin-Ser-Val-Gln-Val-Ser substrate returned no hits.
Since the enzyme databases entries maybe incomplete an extensive literature search within the Pubmed database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&_DB=pumed) performed with the derived information about the substrate specifity and the cleavage sites of Fraction III.

4.4 PART IV- Protein identification of the Fraction I, II and III

4.4.1 Separation of proteins by gel electrophoresis

Fraction I and III were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE of Fraction I yielded a weak protein band at 45 kDa (figure 30). SDS-PAGE of Fraction III yielded a protein band of high molecular weight (figure 31).
Two-dimensional-electrophoresis of Fraction II yielded protein spots of high molecular weight (figure 32).

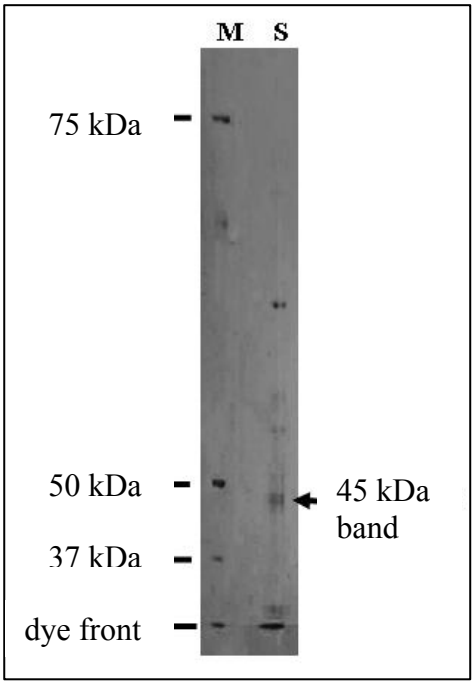


Figure 30 SDS-PAGE of Fraction I.
M: molecular weight marker, S: sample

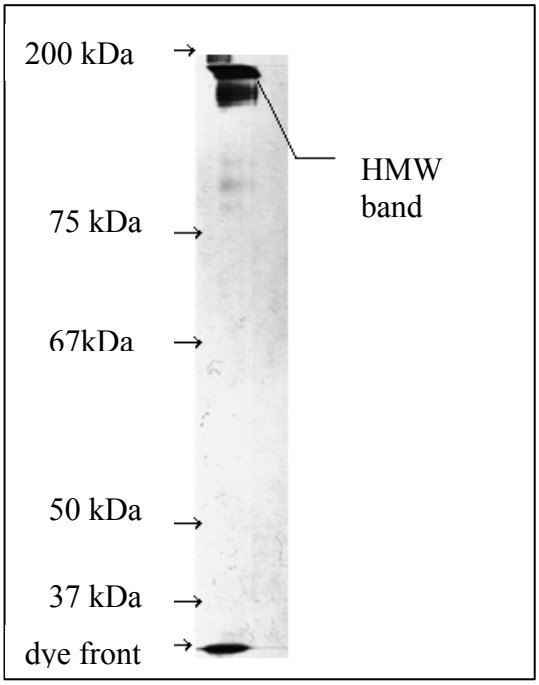


Figure 31 SDS-PAGE of Fraction III.
HMW band: high molecular weight band

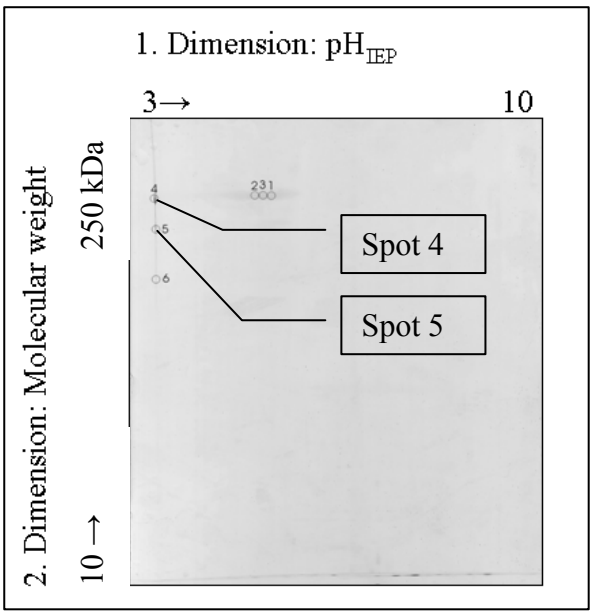


Figure 32 Two dimensional electrophoresis of Fraction II.

4.4.2 In- gel digestion of protein bands with specific endopeptidases

In gel digestion of the protein bands from electrophoresis led to a set of proteolytic peptides. The peptides resulting from the digestion of the protein band were subjected to peptide mass fingerprint experiments.

4.4.3 Peptide-mass fingerprint

The protein spots of fraction II and the high molecular weight protein band of Fraction III could not be identified with the peptide mass fingerprint method.

Figure 33 shows the MALDI-TOF-MS spectra of the proteolytic peptides of the 45 kDa protein band from Fraction I. V8-protease cleaves peptide bonds after the carboxylic termini of the amino acids glutamate and aspartate. Autolytic peptides of the V8-protease were used for internal calibration of the MALDI-TOF-MS spectra. The 45 kDa protein band from Fraction I was identified using peptide mass fingerprint. Autolytic peptides from V8-peptidase were used for internal calibration. Four different peptides could be used for the identification. The monoisotopic peptide masses were entered in the mascot program from Matrixscience® and comparison with the MSDB database porcine Cathepsin G (EC 3.4.21.20) was identified. The corresponding peptide sequences were automatically derived from the Uniprot database using the BLAST-algorithm.

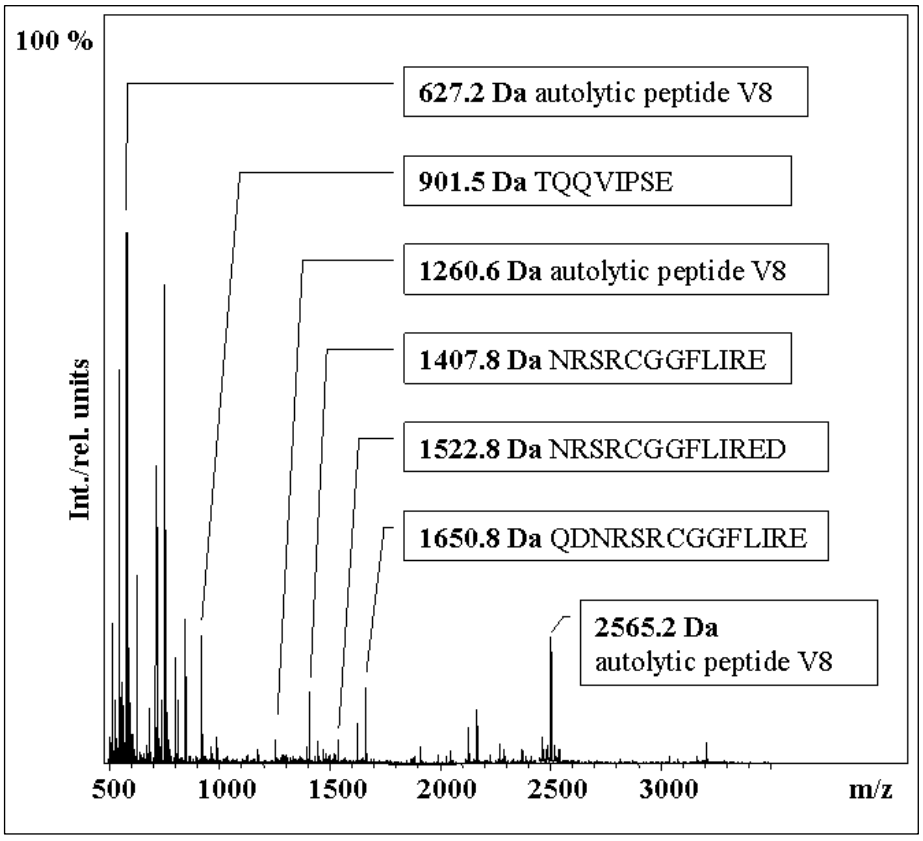


Figure 33 MALDI-MS spectra of the fingerprint peptides of the 45 kDa protein band of Fraction I (figure 30). Molecular masses of peptide peaks and corresponding amino acid sequence given in boxes.

4.4.4 NanoLC-ESI-MS/MS of the proteolytic peptides of Fraction II

Figure 34 shows an exemplary chromatogram of nano Liquid Chromatography (nanoLC) of the proteolytic peptides of Spot 4 and 5 (Figure 32) of Fraction II. The peptides from the enzymatic digestion with trypsin were applied to an ESI-MS/MS experiment. MS^2 analysis of peptide peaks was done and the fragmentation results were compared with MSDB database in order to get amino acid sequences. Table 8 shows the list of the proteolytic peptides and their corresponding masses and the suggested amino acid sequences. The BLAST algorithm was used for homology search within the Uniprot database. Both excised and digested protein spots 4 and 5 yielded the same set of peptide sequences and therefore the same identification. Fraction II was identified by homology as Angiotensin converting enzyme (synonymous peptidyl-dipeptidase A, EC 3.4.15.1). There was no database entry for porcine angiotensin converting enzyme, but the determined sequences showed extensive homology to rabbit ACE (figure 35).

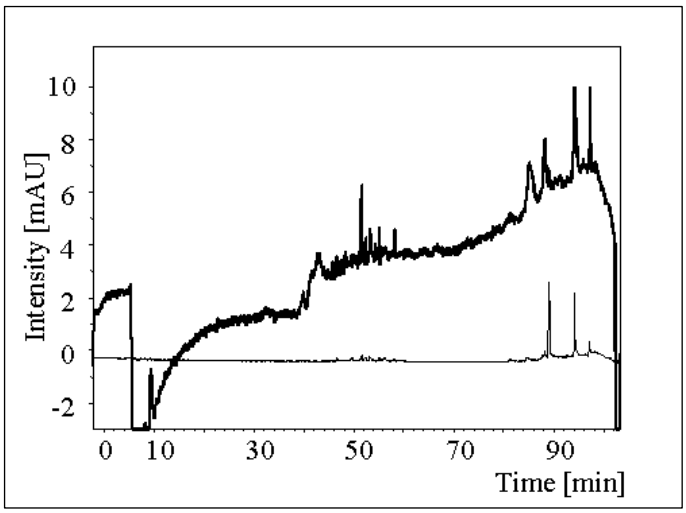


Figure 34 Exemplary Chromatogram of the nanoLC of the proteolytic peptides of spot 4, 5 of Fraction II (figure 32)
Column: Reversed Phase C18
Solid fat line: UV-absorption at λ 214 nm, fine line: UV-absorption at λ 280 nm, x-axis: retention time

Number	Molecular mass	Retention time	Amino acid sequence
1	807.40 Da	58.3 min	WGVFSGR
2	947.55 Da	61.3 min	AILPYFPK
3	956.42 Da	51.5 min	FVEEYDR
4	1020.45 Da	48.9 min	SQGDFDPGAK
5	1148.55 Da	46.8 min	SMLEKPTDGR
6	1351.59 Da	55.8 min	LNGYVDAGSWR
7	1401.59 Da	59.2 min	QDGFSDTGAYWR

Table 8 List of the proteolytic peptides of spot 4 and 5 of Fraction II (figure 32) and their corresponding molecular weights and sequences

Match to: **S35484** Score: **231**

peptidyl-dipeptidase A (EC 3.4.15.1) precursor (Angiotensin Converting Enzyme)
pulmonary splice form - rabbit

Nominal mass (M_r): **149564**; Calculated pI value: **6.11**
NCBI BLAST search of S35484 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Oryctolagus cuniculus

Variable modifications: Oxidation (M),Oxidation (HW)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **5%**

Matched peptides shown in **Bold letters with gray background**

```
   1 MGAAPGRRGP RLLRPPPPLL LLLLLLRPPP AALTLDPGLL PGDFAADEAG
  51 ARLFASSYNS SAEQVLFRST AASWAHDTNI TAENARRQEE EALLSQEFAE
 101 AWGRRLRSSM TRCGRTSPTQ SCAGSSGLCA PWPCQPAPGQ AAADNSLLSN
 151 MSQIYSTGRS ASPTRLPAAW SLDPDLNNIL ASSRSYAMLL FAWEGWHNAV
 201 GIPLKPLYQE FTALSNEAYR QDGFSDTGAY WRSWYDSPTF EEDLERIYHQ
 251 LEPLYLNLHA YVRRVLHRRY GDRYINLRGP IPAHLLGNMW AQSWESIYDM
 301 VVPFPDKPNL DVTSTMVQKG WNATHMFRVA EEFFTSLGLL PMPPEFWAES
 351 MLEKPEDGRE VVCHASAWDF YNRKDFRIKQ CTQVTMDQLS TVHHEMGHVQ
 401 YYLQYKDQPV SLRRANPGFH EAIGDVLALS VSTPAHLHKI GLLDHVTNDT
 451 ESDINYLLKM ALEKIAFLPF GYLVDQWRWG VFSGRTPSSR YNFDWWYLRT
 501 KYQGICPPVV RNETHFDAGA KFHIPSVTPY IRYFVSFVLQ FQFHQALCME
 551 AGHQGPLHQC DIYQSTRAGA KLRAVLQAGC SRPWQEVLKD MVASDALDAQ
 601 PLLDYFQPVT QWLQEQNERN GEVLGWPEYQ WRPPLPNNYP EGIDLVTDEA
 651 EASRFVEEYD RSFQAVWNEY AEANWNYNTN ITTEASKILL QKNMQIANHT
 701 LTYGNWARRF DVSNFQNATS KRIIKKVQDL QRAVLPVKEL EEYNQILLDM
 751 ETIYSVANVC RVDGSCLQLE PDLTNLMATS RKYDELLWVW TSWRDKVGRA
 801 ILPYFPKYVE FTNKAARLNG YVDAGDSWRS MYETPTLEQD LERLFQELQP
 851 LYLNLHAYVG RALHRHYGAQ HINLEGPIPA HLLGNMWAQT WSNIYDLVAP
 901 FPSASTMDAT EAMIKQGWTP RRMFEEADKF FISLGLLPVP PEFWNKSMLE
 951 KPTDGREVVC HASAWDFYNG KDFRIKQCTT VNMEDLVVVH HEMGHIQYFM
1001 QYKDLPVALR EGANPGFHEA IGDVLALSVS TPKHLHSINL LSSEGGGYEH
1051 DINFLMKMAL DKIAFIPFSY LVDEWRWRVF DGSITKENYN QEWWSLRLKY
1101 QGLCPPAPRS QGDFDPGAKF HIPSSVPYIR YFVSFIIQFQ FHEALCKAAG
1151 HTGPLHTCDI YQSKEAGKRL ADAMKLGYSK PWPEAMKVIT GQPNMSASAM
1201 MNYFKPLMDW LLTENGRHGE KLGWPQYTWT PNSARSEGSL PDSGRVNFLG
1251 MNLDAQQARV GQWVLLFLGV ALLLASLGLT QRLFSIRYQS LRQPHHGPQF
1301 GSEVELRHS
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
221 - 232	701.77	1401.53	1401.59	-0.06	0	**QDGFSDTGAYWR**	(Ions score 80)
479 - 485	404.71	807.43	807.40	0.02	0	**WGVFSGR**	(Ions score 42)
655 - 661	479.25	956.49	956.42	0.07	0	**FVEEYDR**	(Ions score 14)
800 - 807	474.79	947.58	947.55	0.03	0	**AILPYFPK**	(Ions score 33)
818 - 829	676.75	1351.49	1351.62	-0.13	0	**LNGYVDAGDSWR**	(Ions score 24)
947 - 956	383.87	1148.60	1148.55	0.05	0	**SMLEKPTDGR**	(Ions score 10)
1110 - 1119	511.24	1020.47	1020.45	0.02	0	**SQGDFDPGAK**	(Ions score 31)

Figure 35 Uniprot entry of the matched peptides form the proteolytic digestion of spot 4 and 5 of Fraction II (figure 32). Amino acid sequence matching was done using the BLAST algorithm.

4.4.5 NanoLC-ESI-MS/MS of the proteolytic peptides of Fraction III

The peptides from the enzymatic digestion of the high molecular weight protein band of Fraction III with V8-peptidase were applied to an ESI-MS/MS experiment. MS^2 analysis of the peptide peaks was done and the fragmentation results were compared with MSDB database in order to get amino acid sequences. Table 9 shows the list of the peptides, their corresponding retention time and molecular masses, and their suggested amino acid sequences.

Number	Molecular mass	Retention time	Amino acid sequence
1	1206.25 Da	57 min	GISYVTVTQQE
2	1223.05 Da	63.7 min	VRDLHATVGGAE
3	1320.51 Da	26.6 min	GLIRIDPDGCYVD
4	1649.02	85.6 min	PVFNEK

Table 9 List of the proteolytic peptides of the HMW protein band of Fraction III (figure 31) and their corresponding molecular weights and sequences

The retrieved sequences were entered into the BLAST (*B*asic *l*ocal *a*lignment *s*earch *t*ool) program at the homepage www.ncbi.nlm.nih.gov/BLAST/ of the National Center for Biotechnology Information of the National Institute of Health USA. Using the BLOSUM62 (*BLO*ck *S*coring *M*atrix) algorithm for local alignment the Uniprot database was searched in order to identify similar proteins. The amino acid sequences from purified Fraction III showed no sequence homologies to an entry of an amino acid sequence within the Uniprot database.