1. Introduction

Cardiovascular diseases are one of the main causes of death in the western world and hypertension is the main risk factor for cardiovascular diseases. Hypertension is the permanent increase of the blood pressure in the arterial vessel system. It is clinically defined as having a systolic blood pressure > 140 mm Hg or a diastolic blood pressure > 90 mm Hg, measured on multiple occasions. Blood pressure is the pressure exerted by the blood on the walls of the arteries. It depends on the amount of blood pumped by the heart and the resistance, which is caused mainly by the degree of constriction of the arterioles (1). Blood pressure (*BP*) is a function of cardiac output (*CO*) and the systemic vascular resistance (*SVR*). Cardiac output is the volume of blood the heart can pump in a given amount of time, usually expressed in liters per minute (L/min). Cardiac output is expressed as the product of heart rate (*HR*) measured in "beats per minutes" and stroke volume (*SV*), the volume of blood pumped with each beat. Therefore blood pressure is a function of three variables: $BP=HR \times SV \times SVR$. Hypertension is classified by its probable causation as *primary* (*essential*) and *secondary*. In case of essential hypertension, which occurs in more than 90% of the hypertensive patients diagnosed no ascertainable cause of hypertension is found. About 10% of the hypertensive patients have an identifiable cause of their elevated blood pressure, and hence have secondary hypertension (2). Secondary hypertension is considered as an accompanying result of another disease. Kidney damage and impaired function, such as inflammation and atherosclerosis, account for most secondary forms of hypertension (3).

Although many molecules likely affect blood pressure, the best understood is the renin angiotensin system (RAS). Within this system the glycoprotein angiotensinogen, produced by the liver, is cleaved by renin, an aspartic protease found in the juxtaglomular cells of the kidney, to form the decapeptide angiotensin-I (Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Ile^{5}-His^{6}-Pro^{7}-Phe^{8}-His^{9}-Leu^{10}). Angiotensin-I is then proteolytic cleaved to angiotensin-II (Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Ile^{5}-His^{6}-Pro^{7}-Phe^{8}) by the dipeptidyl- peptidase angiotensin converting enzyme (ACE). The metallo protease angiotensin converting enzyme is the main enzyme to convert angiotensin-I to angiotensin-II (4). The octapeptide angiotensin-II is the physiologically active hormone that plays an important role in the regulation of blood pressure and fluid balance. The physiological activity of angiotensin-II is mediated by binding to specific angiotensin-II-receptors. Today four subtypes of angiotensin-II-receptors are known: AT_1, AT_2, AT_3 and AT_4. The binding of angiotensin-II to the type I angiotensin receptor (AT_1) on the membrane of smooth muscles of the blood vessels leads to vasoconstriction and an increase of blood pressure (5). Angiotensin-II is not the only

physiologically active hormone. Other angiotensin peptides such as angiotensin-III (Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8), angiotensin-IV (Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) and angiotensin (1-7) (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7) are generated from the same precursor protein angiotensinogen by actions of renin, angiotensin converting enzyme and various other carboxy- and amino-peptidases (6). While the decapeptide angiotensin-I is considered inactive, angiotensin-II and angiotensin-III are full agonists of the type I (AT_1) and type II (AT_2) angiotensin receptor. Binding to the AT_1 receptor mediates major actions such as vasoconstriction, fluid balance, vascular and cardiac cell growth. Li et al. (7) proved that the vasoconstriction in rat aorta induced by angiotensin-II was four times stronger than compared to angiotensin-III. Studies indicate that angiotensin-III is the main effector peptide of the brain renin-angiotensin-system in the regulation of blood pressure (8). Binding to the AT_2 receptor mediates major actions such as vasodilatation, inhibition of cellular proliferation and induction of apoptosis (6). Angiotensin-IV and angiotensin 1-7 display lower affinity for AT_1 and AT_2 receptors. Angiotensin-IV induced vasoconstriction as well as vasodilatation. The binding of angiotensin-IV to the AT_4-receptor also promoted an increased blood flow in the brain (6). The receptor for angiotensin-IV was purified and named type IV angiotensin receptor (AT_4) (9). Components of the renin-angiotensin-system have a widespread tissue distribution. The angiotensin peptides are not only blood-borne hormones that are produced and act in the circulation but they are also having functions as paracrine and autocrine hormone in tissues such as brain (10), heart (11) and blood vessels (12).

Cornish et al. (13) described an ACE-independent way of angiotensin-II formation in the coronary arteries of hamsters. He noticed that the vasoconstriction induced by angiotensin-II was just partially blocked in presence of inhibitors of angiotensin converting enzyme. Contrary the vasoconstriction was completely blocked in presence of an antagonist of the AT_1-receptor. Following studies proved the existence of several ACE-independent angiotensin-II-generating enzymes. ACE-inhibitors and serine protease inhibitors alone did just partially block the angiotensin-II-generating activity, while a combination of ACE-inhibitors and serine protease inhibitors caused a complete block of the angiotensin-II synthesis (13, 14). Urata et al. (15) identified in vitro an ACE-independent pathway for angiotensin-II formation in the human heart and vascular walls. He discovered that about 75% of the angiotensin-II production is mediated by an unknown serine protease. This protease was purified from heart tissue and classified into the chymase family and named cardiac chymase. Later chymase-like enzyme activities were detected in other tissues such as skin, lung and kidney (16). The following research proved that

the result obtained from in vitro experiments also had importance in vivo. Hollenberg (17) showed that a complete block of the renin-angiotensin-system in heart tissue was not obtained by a treatment with an ACE-blocking drug. In a series of in vivo experiments healthy probands were treated with three different ACE-blocking drugs, two renin-blocking drugs and two angiotensin-II receptor antagonists. The reduction of renal blood pressure was 50 % more effective in presence of renin-blocking drugs and angiotensin-II receptor antagonist than in presence of ACE-blocking drugs. Further experiments proved the existence of many other ACE-independent angiotensin-II forming serine proteases (18). The most renin- and ACE-independent angiotensin-I and /or angiotensin-II-generating enzymes are distinguished by their substrate specifity: renin-like proteases which convert angiotensinogen to angiotensin-I, ACE-like proteases which convert angiotensin-I to angiotensin-II and proteases which can use either angiotensinogen or angiotensin-I for the formation of angiotensin-II; in the case that angiotensinogen is converted to angiotensin-II no intermediate angiotensin-I is formed. The following table 1 gives an overview of the angiotensin-I and angiotensin-II-generating enzymes (taken from (19)).

Enzyme	Reaction	Enzyme classification
ACE (20)	A-I→A-II A-I-des-Leu (1-9)→A-II	carboxy peptidase
ACE2 (21, 22)	A-I→ A-I-des-Leu (1-9)	carboxy peptidase
Cathepsin A (23)	A-I→ A-I-des-Leu (1-9) A-I-des-Leu (1-9)→A-II	carboxy peptidase
Cathepsin B (24)	A-I→A-II	carboxy peptidase
Cathepsin D (25)	A→A-I	aspartate protease
Cathepsin G (26, 27)	A-I→A-II A→A-II	serine protease
Chymase (15)	A-I→A-II	serine protease
Enzyme-I (28)	A→A-I	carboxy peptidase
Enzyme-II (29)	A→A-I	carboxy peptidase
Enzyme-III (30)	A→A-II A-I→A-II	serine protease
Kallikrein (31, 32)	A→A-II A-I→A-II	serine protease
KLP-S3 (33)	A-I→A-II	serine protease
Renin (34, 35)	A→A-I	aspartate protease
Tonin (36)	A→A-II A-I→A-II	serine protease
Trypsin (37)	A→A-II	serine protease

Table 1 Overview of the angiotensin-I and angiotensin-II-generating enzymes, A: angiotensinogen, A-I: angiotensin-I, A-II: angiotensin-II, A-I-des-Leu (1-9): Angiotensin-I-des-Leu (1-9)

Arakawa (38) suggested a classification of angiotensin-II-generating serine protease into first: aprotinin-sensitive or kallikrein type which forms bradykinin as well as angiotensin-II. This may contribute to the regulation of tissue perfusion and second: chymostatin-sensitive or chymase type which only forms angiotensin-II and may participate in structural remodelling of heart and vessels (39).

Donoghue et al. (21) discovered ACE2, the first known human homologue of the angiotensin converting enzyme. It was found in heart, kidney and testis of humans. ACE and ACE2 are both carboxy peptidases. They catalyze the shortening of peptides from their carboxy-terminal end. Whereas ACE cleaves two amino acids at time to convert the decapeptide angiotensin-I to the

octapeptide angiotensin-II, ACE2 shortens the angiotensin-I decapeptide by only one amino acid to the nonapeptide angiotensin-I-des-Leu (1-9). Whereas Angiotensin-II stimulates blood vessel constriction and raises blood pressure angiotensin-I-des-Leu (1-9) has no known effect. Angiotensin-I-des-Leu (1-9) cannot be converted to angiotensin-II by ACE but it is converted to angiotensin (1-7) (a blood vessel dilator) by ACE. Crackower et al. (39) found out that ACE2-knockout mice have normal blood pressure but develop abnormal heart function as they age. Specifically the heart muscle develops a significant defect in both speed and strength of contraction.

Other interesting components of the angiotensin-II production are the enzymes of the kallikrein-kinin-system. Kinins such as bradykinin are peptides which play an important role in the regulation of fluid and electrolyte balance, vasodilatation and capillary permeability (40). Kallikrein is an enzyme that converts blood plasma kininogen to bradykinin by proteolytic cleavage. Bradykinin is one of the most potent vasodilators known. ACE inactivates these kinins by proteolytic degradation. Kallikrein-like types of enzymes such as tissue-kallikrein, trypsin and tonin are also capable of using angiotensinogen as well as angiotensin-I as a substrate in order to generate angiotensin-II directly. These enzymes were named kinintensins because of their ability to promote vasoconstriction as well as vasodilatation (37).

Schlüter et al. (41) have developed a new mass-spectrometry-assisted-enzyme-screening (MES)-system for the detection of enzymatic activities. The system involves immobilization of proteins by covalently coupling to activated affinity beads. By immobilization of the proteins the autolytic and proteolytic degradation is prevented. Furthermore molecules that would interfere with the mass spectrometric detection of the enzymatic products are removed. The immobilized proteins are incubated with reaction specific probes. The reaction products were analyzed by matrix-assisted laser desorption ionization (MALDI) -mass spectrometry. Using the MES-system reactions catalyzed by proteases such as angiotensin converting enzyme, kallikrein, renin and urotensin converting enzyme could be. The experiments also showed that the MALDI-MES method allows sufficient quantification of reaction products enabling the detection effect of enzyme inhibitors on enzyme activities.

Belgardt (19) proved that the MES-system can be used for the detection of angiotensin-I and angiotensin-II-generating enzymes in porcine renal tissue. ACE-and renin-independent

endopeptidases that are able to use angiotensinogen and /or angiotensin-I as a substrate were monitored with the MES-system.

2. The aim of the study

Several results point to the existence of proteases other than angiotensin converting enzyme and renin, which significantly generate angiotensin-II. The objective of this work is the purification, characterization and identification of those angiotensin-II-generating enzymes from porcine renal tissue.

The strategy to identify angiotensin-II-generating activities comprises three steps:

1) The MES-assay system is used to detect the angiotensin-II-generating activity. Proteolytic activities are characterized with the help of specific inhibitors.
2) The proteases are purified to near homogeneity by using chromatographic methods.
3) The purified enzymes are identified using enzymatic digestion coupled with mass spectrometry techniques. By using sequence alignment algorithm and protein sequence databases homologies to other enzymes are explored.