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Automated multi-step purification protocol for Angiotensin-I-Converting-Enzyme (ACE)

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ABSTRACT

Highly purified proteins are essential for the investigation of the functional and biochemical properties of proteins. The purification of a protein requires several steps, which are often time-consuming. In our study, the Angiotensin-I-Converting-Enzyme (ACE; EC 3.4.15.1) was solubilised from pig lung without additional detergents, which are commonly used, under mild alkaline conditions in a Tris–HCl buffer (50 mM, pH 9.0) for 48 h. An automation of the ACE purification was performed using a multi-step protocol in less than 8 h, resulting in a purified protein with a specific activity of 37 U mg^{-1} (purification factor 308) and a yield of 23.6%. The automated ACE purification used an ordinary fast-protein-liquid-chromatography (FPLC) system equipped with two additional switching valves. These switching valves were needed for the buffer stream inversion and for the connection of the Superloop™ used for the protein parking. Automated ACE purification was performed using four combined chromatography steps, including two desalting procedures. The purification methods contained two hydrophobic interaction chromatography steps, a Cibacron 3FG-A chromatography step and a strong anion exchange chromatography step. The purified ACE was characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE. The estimated monomer size of the purified glycosylated ACE was determined to be ~175 kDa by SDS-PAGE, with the dimeric form at ~330 kDa as characterised by a native PAGE using a novel activity staining protocol. For the activity staining, the tripeptide L-Phe-Gly-Gly was used as the substrate. The ACE cleaved the dipeptide Gly-Gly, releasing the L-Phe to be oxidised with L-amino acid oxidase. Combined with peroxidase and *o*-dianisidine, the generated H_2O_2 stained a brown coloured band. This automated purification protocol can be easily adapted to be used with other protein purification tasks.

1. Introduction

Angiotensin-I-Converting Enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase found in several mammalian tissues [1]. ACE is a high molecular weight glycosylated integral membrane protein (172 kDa with and 132 kDa without glycosylation), located on the luminal surface of the cell membrane of both endothelial cells and other cell types [2,3]. The three main forms of ACE are somatic, plasma and testicular [4]. Somatic ACE is anchored to the plasma membrane by a C-terminal hydrophobic anchor. Plasma ACE is derived from the somatic form by the cleavage of the hydrophobic anchor in the C-terminal region. The circulating plasma ACE lacks the transmembrane and intracellular domains, but retains the two catalytic sites [5]. ACE plays a major role in blood pressure regulation by catalysing the conversion of the decapeptide angiotensin I to the octapeptide angiotensin II, a potent pressor hormone [6]. ACE also hydrolyses bradykinin, a vasodilating peptide [7].

The recognition of the role of ACE in the blood regulation system has stimulated interest in using ACE inhibitors as antihypertensive agents [4]. To characterise the catalytic process and to develop biologically active inhibitors of ACE, it is necessary to produce purified ACE. Efficient solubilisation of the biologically intact ACE is one of the most difficult problems in the purification of ACE [2]. Detergents such as Triton-X-100, Nonidet-P40 or 1-O-n-octyl-β-D-glucopyranoside have been used for the solubilisation of ACE [4,8,9]. Triton-X-100 or Nonidet-P40 are often used for the solubilisation of membrane proteins, but due to their high absorbance at 228 nm these detergents can interfere with the ACE assay procedures, making the calculation of the ACE purification yields difficult [2,9]. Many investigators prefer the solubilisation of ACE with trypsin. In this process, trypsin cleaves the membrane binding site of ACE, removing the membrane anchor [2].

A major problem in the ACE purification from tissue materials is the separation of ACE from the other proteolytic enzymes that hydrolyse ACE and its substrate, angiotensin I, as well as the

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product angiotensin II [2]. Early attempts at developing ACE purification procedures included heating at high temperatures (60 °C) to inactivate the angiotensinases [10]. The crude lung homogenisate has also been acidified and processed using an ammonium sulphate fractionation [11]. Several multi-step chromatographic procedures, containing anion exchange and size exclusion chromatography, were also developed for the purification of ACE. One substantial improvement in the purification of ACE was the development of a substrate and an inhibitor affinity chromatography, allowing the preparation of a highly purified ACE in a single chromatographic step [2]. Even with these improvements, the purification of ACE can still be time-consuming, often taking several days to complete [8].

In proteome research, a multidimensional liquid chromatography (MDLC) process coupled to mass spectrometry is used for analytical chromatography due to its high speed, high resolution and high sensitivity [12]. The commercially available MDLC systems are not designed for preparative protein separations, which require large quantities of protein [13]. Bhikhabhai et al. [14] developed an automated preparative chromatography system designed for affinity tagged recombinant proteins. Another multidimensional preparative chromatography system was developed by Kong et al. [13] for the purification of plasma proteins from the human plasma fraction IV. This system had three separate units, each equipped with a pump, a UV-detector and up to four columns. The three units were connected with a main pipeline. A fully automated protocol was also developed to purify recombinant aspartate β-semialdehyde dehydrogenase (ASADH) from *Vibrio cholera* [15], using either an affinity and an ion exchange chromatography process or an ion exchange chromatography combined with a hydrophobic interaction chromatography process.

We report a detergent free solubilisation for ACE, followed by a fast, reproducible and automated purification protocol. Furthermore, a protocol for the activity staining of ACE with the substrate L-Phe-Gly-Gly was developed to analyse the results from a native PAGE.

2. Experimental

2.1. Materials

All analytical grade chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany), Applichem (Darmstadt, Germany) or CarlRoth (Karlsruhe, Germany). The ACE substrates hippuryl-histidyl-leucine and phenylalanine-glycl-glycine were purchased from Bachem AG (Bubendorf, Switzerland). Precast SDS-gels (4–15%) were purchased from BIO-RAD (München, Germany). Toyopearl Phenyl-650M was a kind gift from Tosoh Bioscience (Stuttgart, Germany) and used to fill a XK 16/40 column from GE (München, Germany). The HiPrep™ 26/10 desalting, the Resource™ Phe, the Cibacron Blue 3FG-A and the Superloop™ were ordered from GE (München, Germany). The strong anion exchanger Workbeads 17Q as well as the Bioline chromatography system was a courtesy of Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH (Berlin, Germany). Pig lung was obtained from a local slaughterhouse.

2.2. Angiotensin-I-Converting-Enzyme (ACE) purification

2.2.1. Solubilisation of ACE

Pig lungs were used freshly or after storage at −80 °C. Small pieces of pig lung (50 g) were always mixed with 150 ml of cooled (4 °C) Tris–HCl buffer (10 mM, pH 7.0) containing NaCl (0.25 M). Pig lung was minced in a blender (Braun, Kronberg/Taunus, Germany) for 30 s at the highest speed. The crude extract was centrifuged at 48,700 × *g* at 4 °C for 15 min. The resulting supernatant was discarded and the pellet was always resuspended in 150 ml of a cooled (4 °C) Tris–HCl buffer (10 mM, pH 7.0) containing NaCl (0.25 M) followed by a centrifugation in the same manner as described above. The resulting pellet was resuspended in either a Tris–HCl buffer (50 mM, pH 7.0, 8.0 or 9.0), a glycine–NaOH buffer (50 mM, pH 9.0, 10.0, 11.0 or 12.0), or a Tris–HCl buffer (50 mM, pH 7.0) containing 1% (w/v) Triton-X-100 as the reference. The solubilisation of ACE was processed, unless otherwise mentioned, at 4 °C under agitation (200 rpm) for 48 h. The crude extract was centrifuged at 48,700 × *g* for 15 min and the resulting supernatant (cell free extract) was used for the further investigations.

The solubilisation of ACE was investigated using ultracentrifugation. Therefore, a 250 μl cell free extract solubilised with Tris–HCl (50 mM, pH 9.0) and a 250 μl cell free extract solubilised Tris–HCl buffer (50 mM, pH 7.0) containing 1% (w/v) Triton-X-100 were investigated using ultracentrifugation (Beckmann, TL-100 ultracentrifuge, Krefeld, Germany) at 100,000 × *g* at 4 °C for 1 h. After the ultracentrifugation process, the residual ACE activity of the resulting supernatant was measured with the ACE-assay.

For the automated ACE purification process, the cell free extract was treated to obtain a final concentration of 1.5 M ammonium sulphate. A Tris–HCl buffer (50 mM, pH 8.0) containing ammonium sulphate (3 M) was added slowly at a flow rate of 0.5 ml min^{-1} via a peristaltic pump (Watson-Marlow, Rommerskirchen, Germany) at 4 °C. The mixture was allowed to stand at 4 °C for 1 h. The suspension was then centrifuged (8000 × *g*, 4 °C, 15 min) and filtered using a 0.45 μm filter (cellulose mixed ester; CS-Chromatographie Service GmbH, Langerwehe, Germany).

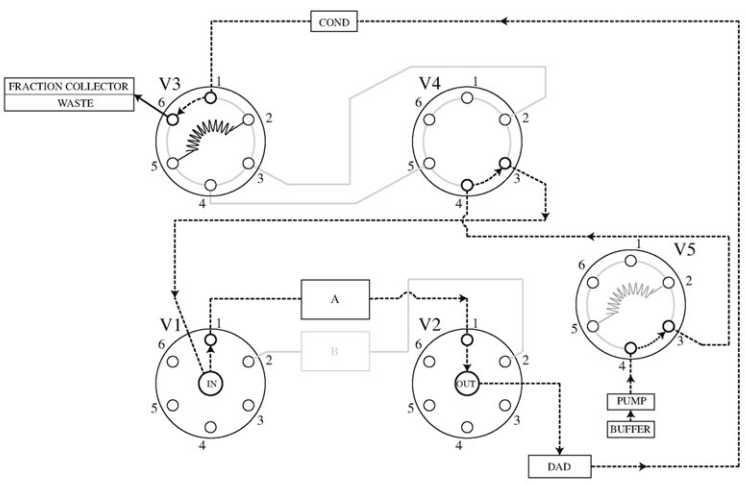
2.2.2. Automated purification of ACE

The ACE solution was purified using a Bioline chromatography system (Knauer, Berlin, Germany; Fig. 1). The system was equipped with a pump (S 1000), a diode array detector (DAD, S 2850), a conductivity meter (S 2900), a manual injection 6-port/3-channel injection valve (V5), two 7-port/1-channel switching valves (V1 and V2) and two 6-port/3-channel injection valves (V3 and V4). The eluting purified ACE was collected using a fraction collector (Frac 3050) in 0.5 ml fractions. The fraction collector was bench top cooled with a Ministat 230 (Huber, Offenburg, Germany) at 4 °C. The Bioline chromatography system was controlled by ChromGate Data System V.3.3.2.

The two 7-port/1-channel switching valves were used for the connection of the chromatography columns to the FPLC system (Fig. 1, V1 and V2). Two 6-port/3-channel switching valves were used for the protein parking and the automated injection of the parked ACE (Fig. 1, V3 and V4). Valve 3 was needed to connect the Superloop™ to the FPLC system. The Superloop™ enabled the ACE parking during the various chromatography steps. Valve 4 enabled the buffer stream inversion of the system, allowing the protein sample (Superloop™) to enter the next column. Sample injections were performed using valve 5.

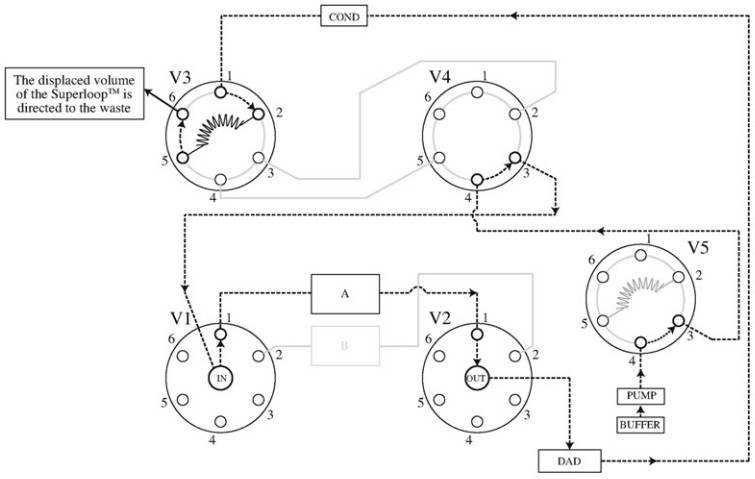
The FPLC system had three alternating valve configurations (Fig. 1a–c). The operating valves were simultaneously switched between the configurations. The initial configuration (the standard configuration of a FPLC system, Fig. 1a) was used when the crude extract was added to the first chromatography column. The protein mixture was manually loaded via valve 5 (switch not shown in Fig. 1a) to start the automated purification. The second system configuration (Fig. 1b) was used when the protein of interest eluted from the operating chromatography column. In this configuration, the protein fraction of interest was subjected to the Superloop™ and parked (a switch of V3 in Fig. 1b). In the third system configuration (Fig. 1c), the Superloop™ volume was injected into the next column via a switch of valve 4. For each chromatography step, the three configurations 1a–c were performed in the order of 1a,

a



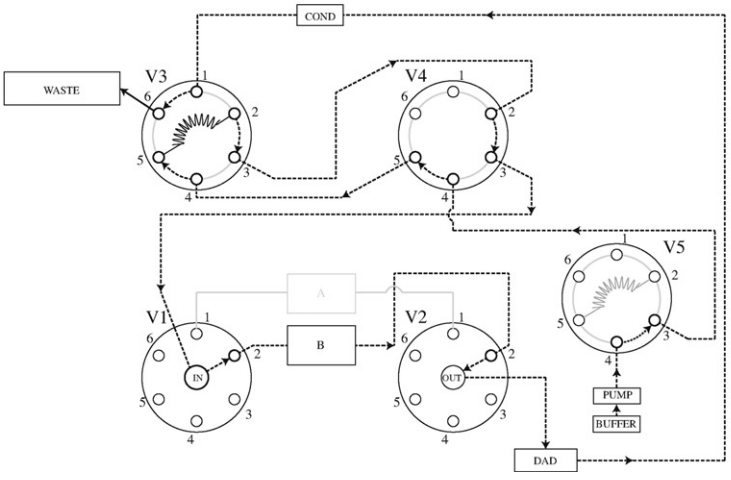
Initial configuration
This position represents the standard FPLC position. Eluting not bound protein is directed to the waste.

b



Protein parking
In this position the protein of interest is directed to the Superloop™ and parked (switch of V3).

c



Automated injection
The previously parked protein is automatically injected to the chromatography column B by buffer stream inversion (switch of V4).

Fig. 1. Flow scheme illustrating the valve position set up for the automated purification of ACE from pig lung (a: the valve position for the standard FPLC; b: the valve position for the protein parking; c: the valve position to inject the previously parked protein; V1–V5: different switching valves; A and B: representative chromatography columns, the number of columns may be increased up to six; DAD: Diode Array Detector; COND: conductivity meter; arrows indicate the flow direction starting with the buffers).

1b, 1a, 1c, and back to 1a. The final chromatography step was performed using the system configuration 1a, where the purified ACE was collected in a fraction collector.

The column matrices, the column volumes (CV), the injected volumes and the buffer systems are summarised in Table 1. To begin the process, a volume of 40 ml of the ACE crude extract was injected via a Superloop™ at a flow rate of 1 ml min^{-1} in a Toyopearl Phenyl-650M (particle size: 40–90 μm) filled column (XK 16/40, 1.6 cm × 40 cm). The unbound protein was washed out at a flow rate of 2 ml min^{-1} for 2 CV. The bound protein was eluted using a linear gradient (5 CV) from 0 to 100% at a flow rate of 2 ml min^{-1}. The eluted ACE was injected into the Superloop™ from 98 to

Table 1
Summary of used chromatography techniques, column matrices, injected volumes and buffer compositions for ACE purification.

Purification technique	Column matrix	Column volume [ml]	Injection volume [ml]	Buffer Composition
HIC	Toyopearls phenyl 650M	20	40	20 mM Tris–HCl, 1.5 M $(NH_4)_2SO_4$, pH 8.0[a] 20 mM Tris–HCl, pH 8.0[b]
Desalting	HiPrep 26/10	53	22	20 mM Tris–HCl, pH 8.0[c]
Affinity[d]	Cibacron Blue 3FG-A	5	22	20 mM Tris–HCl, pH 8.0
AEX	Workbeads 17Q	4.3	40	20 mM Tris–HCl, 0.05 M NaCl, pH 8.0[a] 20 mM Tris–HCl, 1 M NaCl, pH 8.0[b]
Upsalting	HiPrep 26/10	53	5.5	20 mM Tris–HCl, 1.5 M $(NH_4)_2SO_4$, pH 8.0[c]
HIC	Resource™ Phe	1	10	20 mM Tris–HCl, 1.5 M $(NH_4)_2SO_4$, pH 8.0[a] 20 mM Tris–HCl, pH 8.0[b]

HIC, hydrophobic interaction chromatography; AEX, anion exchange chromatography.
[a] Binding buffer.
[b] Elution buffer.
[c] De- or upsalting.
[d] Negative chromatography.

109 min. The ACE solution was then automatically injected at a flow rate of 2 ml min^{-1} to desalt the solution via two HiPrep™ 26/10 desalting columns (2.6 cm × 10 cm, mean particle size: 90 μm) and to remove albumin using a prepacked Cibacron Blue 3FG-A column (1.6 cm × 2.5 cm, particle size: 90 μm). The desalted and albumin-free protein was then injected in the Superloop™ from 145 to 164.9 min. The ACE solution was then purified with a strong anion exchanger (Workbeads 17Q, 8 mm × 85 mm, particle size: 17 μm), injected from the Superloop™ at flow rate of 0.5 ml min^{-1}. The unbound protein was washed out using 5 CV at a flow rate of 0.5 ml min^{-1}. The bound ACE was eluted using 10 CV with a linear gradient from 50 mM NaCl to 500 mM NaCl. The other bound proteins were eluted using a step change up to 1 M NaCl for 1 CV. The eluted ACE was parked into the Superloop™ from 303 to 314 min. The buffer was upsalted to 1.5 M $(NH_4)_2SO_4$ via one HiPrep™ 26/10 desalting column. The ACE solution was completely injected in the Superloop™ from 398.5 to 403.5 min. ACE was finally purified using a Resource™ Phe column (6.4 mm × 30 mm, particle size: 15 μm, monodisperse). The ACE solution was injected at a flow rate of 0.5 ml min^{-1}. The unbound material was washed out using 3 CV of binding buffer. The ACE was then eluted at a flow rate of 0.5 ml min^{-1} with a linear gradient from 0 to 100% of the elution buffer using 24 CV. The purification process of ACE was monitored by a DAD at 280, 254 and 220 nm. The conductivity was measured with a conductivity meter. The eluted ACE was collected in 0.5 ml fractions. All purification steps were performed at room temperature (22 ± 2 °C). The reproducibility of the automated multi-step purification protocol was examined using six individual experiments ($n = 6$). The ACE purification was also performed in the commonly used batch mode, using the same chromatography columns, buffers, flow rates and purification methods as described above for the automated purification of ACE. For this batch experiment, the fractions showing ACE activity that corresponded to the automated peak parked and the de- or up-salted fractions were manually pooled and then treated by dialysis [12 kDa molecular weight cut off (cellulose membrane; Sigma–Aldrich, Schnelldorf, Germany), 24 h, 4 °C] against 10,000 volumes of the binding buffer (Table 1) to mimic the de- or up-salting process. This experiment was performed to compare the automated version of the protocol with the batch mode.

2.3. ACE-assay

The ACE-assay was performed according to the method of Cushman and Cheung [16] with some modifications. The substrate hippuryl-histidyl-leucine (5 mM, HHL) was dissolved in borate buffer (0.1 M, pH 8.3) containing NaCl (0.3 M). The substrate HHL (75 μl) was preincubated at 37 °C for 5 min. The reaction was started with the addition of ACE (5 μl) and stopped by the addition of HCl (1 M, 20 μl). The products hippuric acid (HA) and histidine-leucine (HL) that were released during the hydrolysis were separated by RP-C18 HPLC and detected at 228 nm using a SpectraSYSTEMS™ liquid chromatography system (Thermo Scientific, Dreieich, Germany) equipped with a pump (P 2000), an autosampler (AS 1000) and a UV-VIS-detector (UV 1000). The chromatography system was controlled by ChromQuest 4.2.34. Solvent A consisted of 0.1% (v/v) formic acid (FA) in double-distilled water. Solvent B consisted of 0.1% (v/v) FA in acetonitrile (ACN). The elution of HA and HL was performed with a gradient at a constant flow rate of 0.5 mL min^{-1}. The gradient elution used the following conditions: 0 min (A: 100%, B: 0%); 20 min (A: 50%, B: 50%); 22 min (A: 50%, B: 50%); 28 min (A: 100%, B: 0%); and 35 min (A: 100%, B: 0%). For the HPLC-column, a reversed Phase C18 (Aqua® 5 μm, 200 Å, 150 mm × 4.6 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) was used with a precolumn (Widepore RP C18; 3 μm, 4 mm × 3 mm, Phenomenex, Aschaffenburg, Germany). One unit of the ACE activity was defined as the release of 1 μmol HA per min from the HHL substrate in a borate buffer (0.1 M, pH 8.3) containing NaCl (0.3 M) at 37 °C.

2.4. SDS-PAGE and native PAGE

The purified ACE solutions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 4–15%; BioRad, München, Germany) according to the method of Laemmli [17]. The molecular-weight protein standard mixture was obtained from NEB (Broad Range, 2–212 kDa; Frankfurt, Germany) to be used as a reference. The relevant proteins in the standard mixture were myosin (212 kDa), maltose-binding-protein-β-galactosidase (158 kDa), β-galactosidase (116 kDa), phosphorylase b (97.2 kDa) and serum albumin (66.4 kDa). The protein was measured using the method of Bradford [18]. The gels were silver stained for the protein detection [19]. For the native-PAGE (8%) experiments, a native molecular-weight protein standard mixture (SERVA native marker, liquid mix; SERVA, Heidelberg, Germany) was used as the reference. The relevant proteins in the standard mixture were ferritin (720 kDa), ferritin (450 kDa), urease (272 kDa), lactate dehydrogenase (146 kDa) and albumin (67 kDa). The gels were activity stained to visualise the ACE.

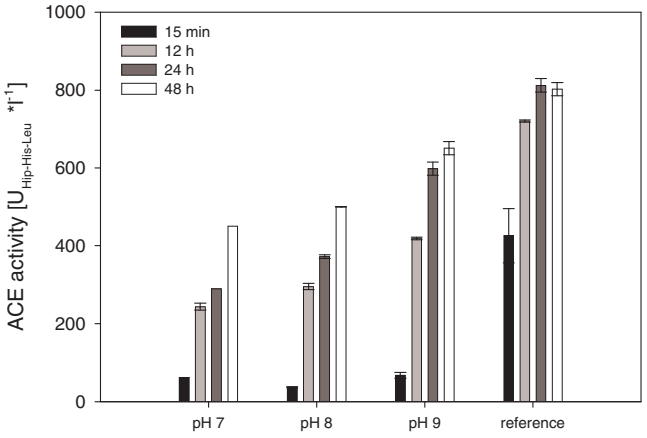


Fig. 2. Dependence of the ACE solubilisation on the pH [Tris–HCl buffer (50 mM)] and time at 4 °C (reference: Tris–HCl buffer (50 mM) containing 1% (w/v) Triton-X-100, pH 7.0).

2.4.1. Activity staining of ACE

The native-PAGE experiments were performed on ice (0 °C). After the electrophoresis, the gels were washed two times with a Tris–HCl buffer (20 mM, pH 8.3). The activity staining was modified from a previously published method [20]. The staining of the ACE activity was performed using L-Phe-Gly-Gly (FGG) as a substrate. The ACE cleaved the dipeptide Gly-Gly, liberating the L-Phe to be used as a substrate for the L-amino acid oxidase (L-AO; Sigma–Aldrich, Schnelldorf, Germany) from rattlesnake (*Crotalus adamanteus*). The L-AO oxidised L-Phe to the corresponding keto-acid, NH_3 and H_2O_2. The generated H_2O_2 was used as the staining molecule, combining with peroxidase (Roche, Mannheim, Germany) and *o*-dianisidine to produce a brown coloured band. For the activity staining, reagents A and B (see below) were mixed and added to the gel. After a brown band had appeared, a solution of 50% (w/v) glycerol was added to prevent the diffusion of the developed brown dye. Reagent A consisted of a Tris–HCl buffer (3 mL; 20 mM, pH 8.3) containing *o*-dianisidine hydrochloride (2 mg), FGG (60 mg), L-AO (8.0 nkat) and peroxidase (4000 nkat). Reagent A was prepared daily. Reagent B consisted of a Tris–HCl buffer (3 ml, 20 mM, pH 8.3) containing agarose [2% (w/v), SERVA, Heidelberg, Germany]. Reagent B was dissolved in a microwave (15 s, 600 W) and cooled to 55–60 °C before being added to reagent A.

3. Results and discussion

3.1. Solubilisation of Angiotensin-I-Converting-Enzyme (ACE) from pig lung

The enzyme ACE has been solubilised with multiple detergents, including Triton-X-100, Nonidet-P40 or octylglucoside [4,8,9], and in combination with sonification [21]. These commonly used detergents, such as Triton-X-100 or Nonidet-P40, are often incompatible with spectrophotometric assays due to their absorbance at 228 nm [9]. For our experiments using Triton-X-100 for the solubilisation of ACE, the detergent was separated from the product hippuric acid using a RP-C18 chromatography column, preventing the interference with the HPLC-assay. The solubilisation of ACE from pig lung without the addition of detergents was also investigated. The solubilisation of ACE from pig lung was investigated for the pH values of 7.0, 8.0 and 9.0 using a Tris–HCl buffer (50 mM) at 4 °C over a period of 48 h (Fig. 2). A longer solubilisation time was prohibitive as lung tissue continues to further degrade into a high viscous liquid that prevents the proper handling of the material. For the solubilisation of ACE under neutral or mildly alkaline conditions, the highest volumetric ACE enzyme activities were measured after 48 h. The measured ACE activities were 651 U l^{-1} (100%) at pH 9.0, 500 U l^{-1} (77%) at pH 8.0 and 450 U l^{-1} (69%) at pH 7.0.

As a reference, the enzyme ACE was also solubilised in the same Tris–HCl buffer (50 mM, pH 7.0) using 1% (w/v) Triton-X-100. The solubilisation of ACE was significantly faster in the presence of the detergent than without the detergent. After a 15 min solubilisation time, an activity of 426 U l^{-1} was detected in the supernatant. The maximum ACE activity of 812 U l^{-1} was achieved after 24 h. Compared with the best solubilisation result without the detergent at pH 9.0, approximately 25% more volumetric enzyme activity was obtained using the detergent method.

For the specific activities, the reference produced 0.184 U mg^{-1} shortly after the detergent addition (15 min). With increasing the solubilisation time, the specific ACE activity decreased with the increased solubilisation of other lung proteins. After 12 h of the solubilisation, the specific ACE activity of 0.147 U mg^{-1} was determined, followed by 0.115 U mg^{-1} (24 h) and 0.103 U mg^{-1} (48 h). The specific ACE activity measured at pH 9.0 (without detergent) increased from 0.055 U mg^{-1} (15 min) to 0.114 U mg^{-1} (12 h), decreasing to 0.09 U mg^{-1} and 0.087 U mg^{-1} after 24 and 48 h, respectively.

The effect of pH (9.0, 10.0, 11.0 and 12.0) on the ACE solubilisation without detergents in a glycine–NaOH buffer (50 mM) was also investigated. The highest activity yields were still measured at pH 9.0 using the Tris–HCl buffer (data not shown). For the remainder of the experiments, the solubilisation of ACE was performed in a Tris–HCl buffer at pH 9.0 and for 48 h.

To prove that ACE was truly solubilised, the crude extract with (reference) and without detergent was measured after an ultracentrifugation (100,000 × *g*; 4 °C; 1 h) process. After the ultracentrifugation, only the solubilised ACE would remain in the supernatant, with the suspended ACE collecting in the pellet. In both preparations, the major ACE activity was found in the supernatant. For the reference, the activity was 91% and the detergent-free extract activity was 78%. These data suggest that the ACE was truly solubilised in both preparations.

3.2. Automated purification of ACE from pig lung

An automated multi-step preparative chromatography protocol was developed for the purification of ACE. The automated chromatographic purification (Fig. 3) started with loading the ACE crude extract on a polydisperse Tosoh Phenyl-650M hydrophobic interaction chromatography (HIC) column (section A in Fig. 3). The ACE fraction eluting between 98 and 109 min was parked in the Superloop™ until further purification. In section B (Fig. 3), a desalting step was directly followed by a Cibacron Blue 3FG-A chromatography step to further separate the proteins. For this purpose the columns were run in sequence. The complete volume of the protein peak between 145 and 165 min was parked in a Superloop™ further purified by subjecting it on a strong anion exchanger (section C in Fig. 3). The ACE fraction eluting between 303 and 314 min was also collected in the Superloop™. This ACE fraction was loaded onto a HiPrep™ 26/10 column (section D in Fig. 3) equilibrated with 1.5 M $(NH_4)_2SO_4$ to increase the ionic strength of the protein sample prior to the HIC step. The complete volume of the protein peak between 398.5 and 403.5 min was parked in the Superloop™ in preparation for the final HIC step (section E in Fig. 3). This HIC step was performed using a monodisperse Resource™ Phe material possessing high-class separation properties. The 1.5 ml fraction shown in the zoomed segment of section E (Fig. 3) was collected and contained the purified ACE. This purified ACE was analysed using both SDS- and native PAGE electrophoresis with the activity staining process described below.

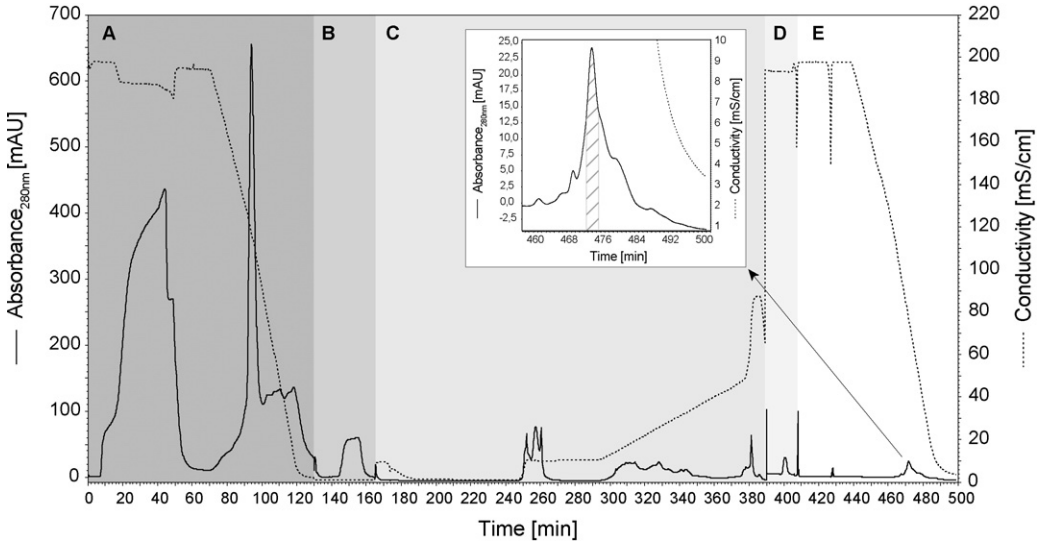


Fig. 3. Chromatogram of the automated multi-step ACE purification protocol (areas A–E stand for the different FPLC techniques; (A) Tosoh Phenyl-650M; (B) two HiPrep™ 26/10 columns (desalting) connected in series with the Cibacron Blue 3FG-A; (C) Workbeads 17Q (strong anion exchange chromatography); (D) HiPrep™ 26/10 column (up-salting); (E) Resource™ Phe shown in detail from 456 to 500 min; pooled fractions are hatched).

The results from the automated multi-step chromatography process used to purify ACE from pig lung are summarised in Table 2. For comparison, the automated purification protocol of ACE was also performed in a manual mode, using the same chromatography methods developed for the automated purification protocol of ACE with the de- and up-salting processes performed by dialysis. With the added dialysis steps, this conventional purification procedure took 3 days, giving similar results to the automated process with a yield of approximately 23% and a specific activity of about 37 U mg^{-1}. These results are consistent with the reported values by Meng et al. [22] for purified ACE from human lung producing a specific activity of ≈40 U mg^{-1} and a yield of 22%. Other ACE purification protocols including dialysis steps [8,23,24] or affinity columns using low flow rates [4,8,23] took up to six days [8]. Compared with these other procedures, our automated purification protocol for ACE was extremely time efficient with a total purification of less than 8 h. Six independent experiments using the automated protocol demonstrated a high reproducibility (yields 25 ± 2%; specific activities 37.0 ± 0.5 U mg^{-1}).

This automated protein purification procedure is also suitable for the purification of multiple types of proteins. This protein purification protocol is compatible with all purification methods, including hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEX), size exclusion chromatography (SEC), immobilised metal affinity chromatography (IMAC) and substrate/inhibitor chromatography. This process may even increase the yields of active proteins, as the long purification times used in the other protocols may have resulted in the inactivation of highly sensitive proteins.

3.3. SDS-PAGE and native PAGE with an activity staining for ACE

The purification of ACE from pig lung was assessed by SDS-PAGE and silver staining (Fig. 4). The monomeric ACE was determined to be approximately 175 kDa, consistent with the estimated size of 172 kDa of the glycosylated ACE from pig lung [3,4].

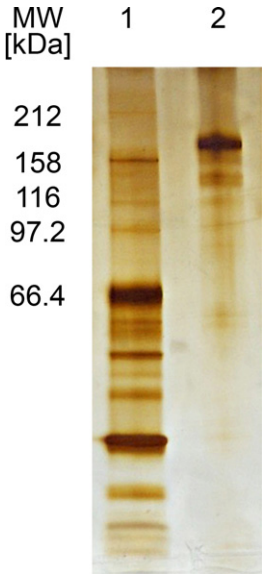


Fig. 4. SDS-PAGE with a silver staining of the automatically purified ACE from pig lung (Lane 1: Molecular weight marker; Lane 2: ACE from pig lung).

The molecular weight was also estimated for the soluble automated purified ACE under non-denaturing conditions by native-PAGE with an activity staining (Fig. 5). The activity stained ACE band appeared at ~330 kDa, consistent with the reported values for the molecular size of human ACE of 300 kDa measured using native PAGE [22]. The differences between the molecular weight values obtained by the SDS-PAGE and the native-PAGE experi-

Table 2
Purification of Angiotensin-I-Converting Enzyme from pig lung.

Fractionation step	Total volume [ml]	Specific activity [U mg^{-1}]	Total protein [mg]	Total activity [U]	Purification (*n*-fold)	Yield (%)
Crude extract	20	0.12	90.2	11	1	100
Purified ACE	1.5	36.9	0.07	2.6	308	23.6

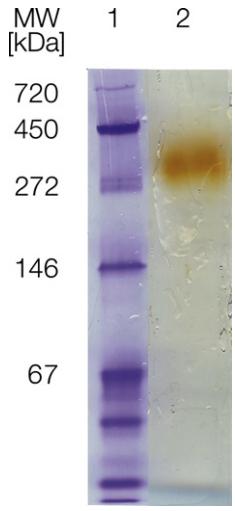


Fig. 5. Native PAGE with an activity staining of the automatically purified ACE from pig lung (Lane 1: Molecular weight marker (Coomassie stained); Lane 2: ACE from pig lung (activity stained)).

ments might be attributed to the hydrodynamic properties of the glycosylated ACE [4]. In addition, a dimerisation of the somatic bovine ACE was also demonstrated in a biomembrane modelling system via carbohydrate controlled interactions [25]. A dimerisation via carbohydrate interactions might be responsible for the differences between the values measured using SDS-PAGE and native-PAGE.

4. Conclusions

The solubilisation of ACE from minced pig lung without the use of detergents was demonstrated for alkaline conditions at pH 9.0 for 48 h. Compared with the process using detergents, the ACE was solubilised with an 80% yield. The solubilised ACE was purified with a novel automated multi-step chromatography protocol in less than 8 h using multiple separation methods in sequence. The automated protocol provided several advantages, including a reduction in the processing time, a purified product with high reproducibility and a reduction in the manpower needed to produce the purified ACE.

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