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Application of cross-flow filtration to the purification of biologically active peptides in human plasma after incubation with a protease-rich extract

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ABSTRACT

The aim of this study was to find an experimental procedure to purify biologically active peptides from a complex biological matrix (plasma), which was incubated with a protease-rich extract (submandibular gland extract). Special interest was focused on the practicability of cross-flow filtration for this purpose. Therefore, peptides in the incubation mixture were purified with a combination of high-performance liquid chromatographic steps. Purification of biologically active peptides was monitored by a sensitive bioassay and by laser desorption/ionization mass spectrometry. This permitted not only purity control at each purification step but also identification of one of the peptides with vasoconstrictor properties as angiotensin II. This result demonstrates the practicability of cross-flow filtration for extracting enzymatic reaction products from complex substrate–enzyme mixtures during the incubation.

INTRODUCTION

Croxatto [1] recently suggested that there may be many unidentified, biologically active peptides released from their precursor proteins by limited enzymatic proteolysis. With his idea in mind, a method for the purification of biologically active peptides was devised. Limited enzymatic proteolysis of precursor proteins, to obtain bioactive peptides, demands the rapid removal of the proteolysis products from the incubation mixture on their generation in order to avoid further cleavage into biologically inactive peptides. The conventional solution for this problem, dialysis [2], seems to be unsatisfactory, as the removal of the peptides is based on diffusion and therefore possibly too slow. Another disadvantage is that dialysis is time consuming and the handling is cumbersome. Cross-flow filtration appears to be a better technique for removing the newly generated peptides from the incubation medium, because the filtration rate is larger, the reaction medium is moved continuously, the risk of membrane clogging is low and the temperature from the retentate and the filtrate can be controlled separately. The practicability of the cross-flow filtration had to be proved by purification and identification of the bioactive peptides from the filtrate.

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Rat submandibular gland tissue was chosen, because it contains a broad spectrum of proteases [3]. Human plasma was chosen as a source of precursor hormones, because it is well documented that several prehormones, such as angiotensinogen, are present in plasma [4]. It was not attempted to demonstrate a mechanism which is active *in vivo*, as this would have required substrate and enzymes from the same species. Further, in this work, the submandibular gland proteins releasing vasoactive peptides were not isolated and identified, as a variety of such submandibular gland enzymes have already been described, such as tonin, kallikrein and other enzymes [5–7].

The background of this study was to find and characterize still unknown peptides with vasoactive properties that may be involved in blood pressure regulation like the peptide hormones angiotensin II, endothelin or bradykinin. So far there is no evidence that the known pressure-regulating peptide hormones are involved in the pathogenesis of hypertension [8,9]. This suggests that other, still unknown, peptide hormones may be involved.

EXPERIMENTAL

Materials

Chemicals. High-performance liquid chromatography (HPLC)-grade water, methanol, acetonitrile and HPLC-grade trifluoroacetic acid (TFA) were obtained from J. T. Baker (Deventer, Netherlands). Angiotensin II (AII), norephinephrine (NE) and AII inhibitor ([Sar^1,Val^5,Ala^8]-AII) were obtained from Sigma (Deisenhofen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Physiological buffer. The buffer was 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 100 m*M* NaOH, 90 m*M* NaCl, 5 m*M* KCl, 1.8 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ dissolved in water and titrated to pH 7.4.

Filtration equipment. Tubular dialysis membranes with an exclusion pore diameter of relative molecular mass (M_r) 8000 were obtained from Spectra/Por (Spectrum Medical, Los Angeles, CA, USA). The cross-flow filtration device and the M_r 10 000 membrane (regenerated cellulose) were purchased from Millipore (Bedford, MA, USA).

SDS-PAGE equipment. The Phast system and PhastGel (gradient 8–25) from Pharmacia Biosystems (Freiburg, Germany) were used.

Solid-phase extraction (SPE) equipment. The vacuum manifold column processor was obtained from Burdick & Jackson (Muskegon, MI, USA), glass columns (5 ml), PTFE frits and reversed-phase (RP) C_{18} silica material from J. T. Baker (Phillipsburg, NJ, USA) and a Speed-Vac concentrator from Savant (Farmingdale, NY, USA).

HPLC equipment. The HPLC equipment consisted of an L-6200 gradient pump (Merck) coupled to a Rheodyne injector (Latek, Heidelberg, Germany), a spectrophotometer (Lambda-Max 481; Waters), a two-channel compensation recorder (Pharmacia Biosystems, Freiburg, Germany) and a RediFrac fraction collector (Pharmacia Biosystems, Freiburg, Germany). A semi-preparative reversed-phase column, Protein Plus (25 cm × 2.2 cm I.D.; Zorbax) (DuPont, Dreieich, Germany) was used.

Microbore HPLC equipment. Microbore separations were performed on the SMART system (Pharmacia Biosystems), which integrates a gradient pump, injector, column holder, a spectrophotometer (280 nm), a flow cell for conductivity measurement and a fraction collector. A microbore cation-exchange column (100 × 2 mm I.D., 100-SA; Machery–Nagel, Düren, Germany) was used. The fractions from cation-exchange chromatography were desalted on a reversed-phase microbore HPLC column (RPC C_2/C_{18}, PC 3.2/3; Pharmacia Biosystems). The GROM-SIL 300-ODS-2 microbore reversed-phase column (200 × 2 mm I.D.) was purchased from Grom/Stargroma (Herrenberg, Germany).

HPLC eluents. Eluent A was 0.1% (v/v) TFA in HPLC-grade water, eluent B was 0.1% (v/v) TFA in HPLC-grade acetonitrile and eluent C was 60% (v/v) HPLC-grade acetonitrile in HPLC-grade water. Eluent D was made up of 5 m*M* KH_2PO_4 (pH 3) in 30% (v/v) acetonitrile and eluent E of 5 m*M* KH_2PO_4 (pH 3) in 30% (v/v) acetonitrile and 0.5 *M* KCl. All HPLC eluents were filtered through a 0.2-μm filter (Anotop) (Merck).

Plasma preparation

A plasma pool was prepared by drawing a total of 830 ml of blood from an antecubital vein from sixteen healthy human volunteers (male 9, female 7, age 28 ± 4 years). Blood samples were collected

in chilled polypropylene tubes, containing 1 mg EDTA/ml plasma. This was centrifuged at 2000 *g* for 10 min at 4°C. The resultant plasma (410 ml) was bulked and stirred on ice for 2 min. Aliquots of 25 ml were frozen in dry-ice and then stored under liquid nitrogen.

Comparison experiments: dialysis versus cross-flow filtration

A 10-ml volume of the pooled plasma was divided into two equal parts and treated as follows.

In method I, 5 ml of plasma were dialysed for 24 h at 4°C through tubular dialysis membranes with an exclusion pore diameter of 8000 dalton against a physiological buffer solution.

In method II, the other 5 ml of plasma were diluted to 100 ml with a physiological buffer solution and then filtered in a cross-flow filtration device, using an M_r 10 000 membrane at 4°C until the volume of the filtrate measured 90 ml.

The filtrate from both methods was lyophilized and desalted by gel filtration. The protein concentration was then measured using the Bradford assay [10] and the samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on Phast-Gel gradient 8–25. Samples treated with SDS were processed in the PhastSystem and the gels were developed with the silver staining procedure of Heukeshoven and Dernick [11].

Incubation experiments

Tissue extraction. An 18.6-g amount of rat submandibular glands obtained from normotensive male Wistar rats aged 3 months were cut into small pieces. The tissue was placed in 40 ml of ice-cold physiological buffer and homogenized in an ice-bath-cooled blade homogenizer ten times at intervals of 30 s. The homogenate was centrifuged and the supernatant was filtered in the cross-flow filtration device using a 0.2-μm membrane. The filtrate (35 ml) was used as an incubation extract. The extraction work was carried out at 4°C.

Incubation of plasma with the submandibular gland extract. Freshly prepared submandibular gland extract was mixed with 390 ml of the pooled plasma. This mixture was filtered in the cross-flow filtration device using an M_r 10 000 membrane. During filtration the plasma–submandibular gland extract mixture (retentate) was incubated at 37°C for 20 h. The retentate was kept at a constant volume with a physiological buffer reservoir (37°C). The filtrate was cooled in an ice–water bath separately.

Concentration of peptides in the filtrate. For concentration of peptides, laboratory-assembled RP-SPE cartridges and a vacuum manifold column processor connected to a water-driven vacuum pump were used. Silanized glass columns were fitted with PTFE frits and filled with 2.5 g of reversed-phase C_{18} silica material. Extraction was carried out with two columns connected in series. The solid-phase extraction was preceded by conditioning the columns with 20 ml of eluent B and subsequently washing with 20 ml of eluent A.

The filtrate from the incubation experiment was acidified with TFA up to a concentration of 0.1% (v/v). This eluent was slowly aspirated (1 ml/min) through the RP-SPE column by vacuum, then the column was washed with 20 ml of eluent A. Elution of the peptides was achieved with 20 ml of eluent C. The eluate was concentrated to dryness in a Speed-Vac concentrator. The filtrate from the control experiments was also processed according to this concentration procedure.

Purification of the peptide-rich RP-SPE fraction by HPLC. The peptide-rich RP-SPE fraction from the incubation experiment (plasma with submandibular gland extract) and the two control experiments (plasma alone, submandibular gland extract alone) were chromatographed first on the semi-preparative RP-HPLC column. The conditions and the procedure are given in the caption of Fig. 4.

The vasoactive fraction from semi-preparative RP-HPLC was purified further on a microbore cation-exchange HPLC column (the conditions and procedure are given in the caption of Fig. 6). Before testing the fractions from the cation-exchange chromatography for vasoactivity, they had to be desalted on a reversed-phase microbore HPLC column. After the sample injection of a fraction into the SMART system, the column was washed for 4 min with eluent A at a flow-rate of 100 μl/min. Elution of the peptides was performed with a steep linear gradient from 0 to 60% eluent B in 2 min and with 60% eluent B for 3 min at a flow-rate of 100 μl/min. The Speed-Vac-dried vasoactive fraction from the cation-exchange separation was chromatographed by microbore RP-HPLC (the conditions and procedure are given in the caption of Fig. 8).

Detection of vasoactivity. Male normotensive Wistar Kyoto rats (WK), aged 4–6 weeks, were used. The isolated perfused kidney was prepared as described previously [12]. Briefly, the abdominal cavity was opened and the infrarenal aorta and left renal artery were isolated and cannulated. Immediately after the cannulation, 500 units of heparin sodium were flushed into the aorta and renal artery. Perfusion was started immediately after the cannulation. The left kidney was excised by blunt dissection, excluding the adrenal gland, and mounted in the perfusion system.

The perfusion was performed with a single-pass system at constant flow-rate of 9 ml/min · g body mass with Tyrode's solution kept at 37°C and equilibrated with 5% CO_2 and 96% O_2, as described previously [13]. The prepared fractions and the vasoactive hormones (concentration of angiotensin II 25 ng/ml; concentration of norepinephrine 300 ng/ml) were injected in bolus injections of 150 μl each to test kidney responsiveness. To block the angiotensin II receptors, the isolated kidney was perfused with Tyrode's solution containing 10 μM of the angiotensin II receptor antagonist saralasin.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. The vasoactive fractions were examined by MALDI-MS. A reflector-type time-of-flight mass spectrometer, equipped with a nitrogen laser (337 nm, pulse length 4 ns) was used for ion generation and mass analysis. Details of the MALDI-MS have been reported elsewhere [14]. Briefly, Speed-Vac-dried samples were dissolved in 10 μl of water. A 1-μl volume of this eluent was mixed with 10 μl of an aqueous solution of 0.1 M 2,5-dihydroxybenzoic acid (UV absorption maximum at 337 and 355 nm), representing the UV-absorbing matrix. A 1-μl volume of the final eluent was dripped in and dried on a metallic substrate. Desorption of analyte ions was achieved by laser shots of irradiances in the 10^6–10^7 W/cm^2 range focused to spot sizes typically 50–100 μm in diameter. The spectra were registered with a LeCroy 9400 transient recorder and typically accumulated from ten single laser shots. The total time of measurements, including preparation, was 10–15 min. The results were expressed as M_r/electrical charge of the substance (M_r/z in Figs. 5, 7 and 9). Since with this form of mass spectrometry substances with a single charge are produced, M_r/z is identical with M_r [14].

RESULTS

Comparison of dialysis with cross-flow filtration

The efficiency of cross-flow filtration was first compared with that of dialysis. Pooled human plasma was divided into two parts and one part was fractionated by dialysis and the other by cross-flow filtration. Table I shows the protein concentration, estimated by the Bradford assay [10]. This shows that cross-flow filtration is significantly more effective than dialysis. To obtain further insight into the quality of the fractionation, SDS-PAGE was performed and the results are shown in Fig. 1. In the dialysis filtrate even albumin can be demonstrated, whereas in the cross-flow filtration filtrate no protein bands can be shown.

Incubation experiment

The plasma was incubated with submandibular gland extract in the cross-flow filtration device at 37°C for 20 h. During the whole incubation period the mixture was filtered against a 10 000 M_r membrane to remove the proteolysis products continuously. The filtrate (proteolysis products) were cooled at 0°C.

Concentration of peptides

When the incubation was complete, the peptides in the filtrate were concentrated by solid-phase extraction with a reversed-phase sorbent (RP-SPE).

Detection of vasoactivity

The fractions from the HPLC purification steps were tested for vasoactivity in a bioassay. For this purpose an isolated perfused rat kidney was used. Fig. 2 shows a dose–response curve of the vasocon-

TABLE I

PROTEIN CONCENTRATIONS (DETERMINED WITH THE BRADFORD ASSAY) OF DIFFERENT FRACTIONS FROM HUMAN PLASMA (n = 5)

Fraction	Protein concentration (mean ± standard deviation)
Dialysis, retentate	6.9 ± 0.8 g/dl
Dialysis, filtrate	1.0 ± 0.03 mg/dl
Cross-flow filtration, retentate	7.2 ± 0.6 g/dl
Cross-flow filtration, filtrate	0.0 ± 0.0 mg/dl

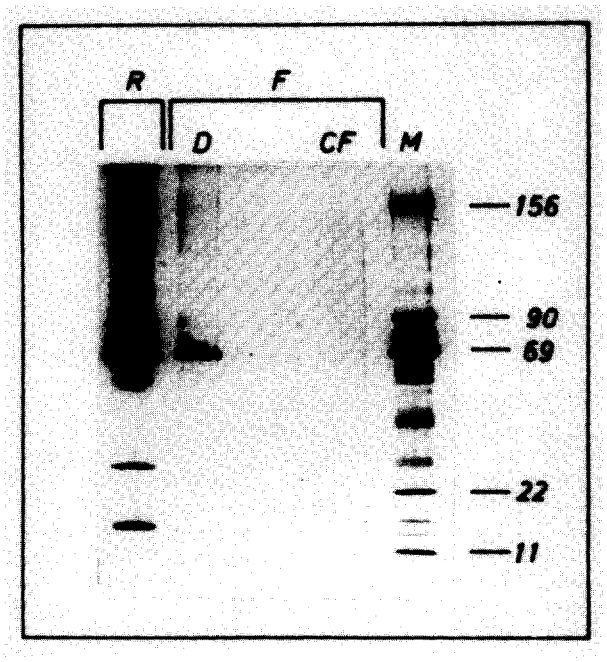

Fig. 1. SDS-PAGE of the plasma fractionation experiments. R = Sample of the retentate; F = samples of the filtrates; D = sample of the filtrate fraction from dialysis; CF = sample of the filtrate fraction from cross-flow filtration; M = marker proteins (desalted human urine proteins); numbers = $M_r \times 10^{-3}$.

strictors angiotensin II and norepinephrine and Fig. 3 an original curve of the time course of the perfusion pressure and the response to vasoactive factors.

Control of the purity of the peptide fractions

MALDI-MS was used to the control the purity of the peptide fractions and to characterize the peptides.

Purification of the vasoactive substance

The peptide-rich SPE-RP fraction was chromatographed first on an RP-HPLC column (Fig. 4). Only in the chromatogram of the incubation experiment (plasma together with submandibular gland extract) was a vasoactive fraction eluted after 34 min. In the fractions from the control experiments (plasma alone, submandibular gland alone) no vasoactivity was registered. The vasoactive fraction from RP-HPLC (Fig. 4) was analysed by MALDI-MS (Fig. 5). Several mass peaks indicate that this fraction is

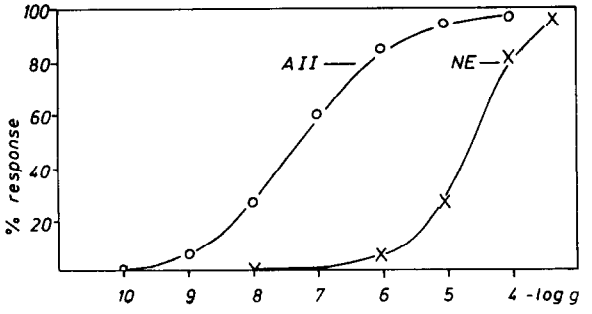

Fig. 2. Dose–response curve of the isolated perfused rat kidney. Abscissa: injected amounts of (○) angiotensin II (AII) and (×) norepinephrine (NE), expressed in −log (grams). Ordinate: change in perfusion pressure (P, mmHg); 100% response of the kidney stimulated with angiotensin II, 90 mmHg; 100% response of the kidney stimulated with norepinephrine, 210 mmHg.

inhomogeneous. One of the peaks has m/z 1047, which is identical with the mass of the protonated ion of angiotensin II. Commercially available angiotensin II, injected to the RP-HPLC column, was eluted at the same retention time as the bioactive fraction.

Next, the vasoactive fraction was purified further with a microbore preparative cation-exchange column (Fig. 6). Again the retention times of the vasoactive fraction and angiotensin II were very similar. The MALDI mass spectrum (Fig. 7) of the vasoactive fraction still shows several mass peaks.

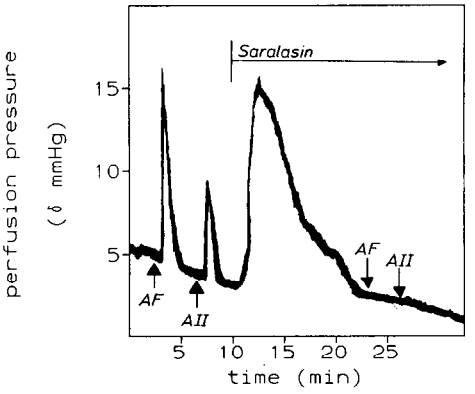

Fig. 3. Typical pattern of changes in perfusion pressure (P, mmHg) in the isolated perfused kidney, caused by 1 ng of angiotensin II (AII) and the bioactive fraction (AF; equivalent to 0.3 ml of plasma) from the chromatographic purification (Fig. 4) before and after adding 10 μM saralasin to the perfusion medium.

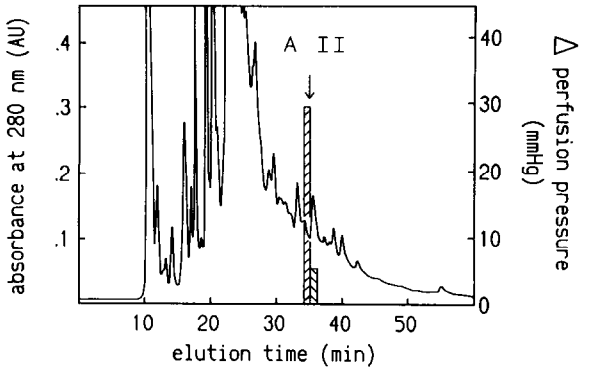

Fig. 4. Typical semi-preparative RP-HPLC of the peptide-rich SPE fraction. AU = Arbitrary units; absorbance range, 0.5. Conditions: column, C_4 reversed-phase Protein Plus (250 × 21.2 mm I.D.); eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile; gradient, 0–5 min 100% eluent A, 5–15 min 0–20% eluent B; 15–55 min 20–40% eluent B; 55–65 min 40–60% eluent B; flow-rate, 6.5 ml/min; sample, peptide-rich SPE fraction (390 ml plasma equivalent), dissolved in 1 ml of eluent A; fraction size, 6.5 ml. The hatched area indicates the vasopressor activity, equivalent to 1 ml of plasma. AII indicates the retention time of angiotensin II.

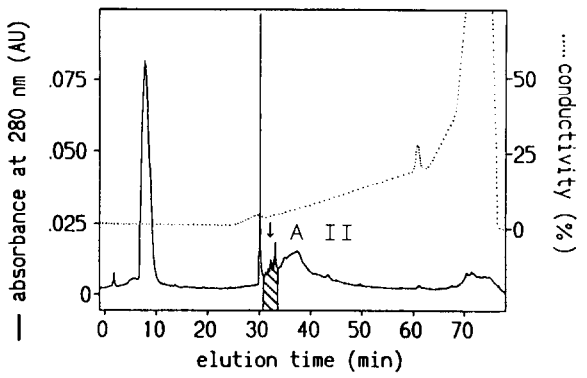

Fig. 6. Typical micropreparative cation-exchange chromatography of the bioactive fraction from the semi-preparative chromatography (Fig. 4). Absorbance range, 0.1. Conditions: column, 100-SA (100 × 2 mm I.D.); eluent D, 5 m*M* KH_2PO_4 (pH 3) in water–30% acetonitrile; eluent E, eluent D + 0.5 *M* KCl; gradient, 0–7.5 min 100% eluent D, 7.5–47.5 min 0–20% eluent E; 47.5–62.5 min 20–35% eluent E, 62.5–64.5 min 35–80% eluent E, 64.5–67.5 min 80% eluent E; flow-rate, 100 μl/min; sample, bioactive fraction from the semi-preparative chromatography (Fig. 4; 350 ml plasma equivalent) dissolved in 1 ml of eluent D; fraction size, 600 μl. The vasoactive fraction, equivalent to 1 ml of plasma, caused an elevation of perfusion pressure of 11 mmHg (hatched area) in the bioassay. AII indicates the retention time of angiotensin II.

One of the mass peaks is identical with the mass of the protonated ion of angiotensin II.

The vasoactive fraction was re-chromatographed on a preparative microbore HPLC column, using a less steep gradient (Fig. 8). The fraction was separated into several UV-absorbing peaks (Fig. 8). Vasoactivity was eluted with the peak at 31 min. The vasoactive fraction and angiotensin II were eluted with the same retention time.

Pharmacological proof of the identity of the purified vasoactive peptide with angiotensin II

To confirm that the vasoactivity is due to angiotensin II, the bioactive fraction from semi-preparative RP-HPLC (Fig. 4) was tested in the isolated,

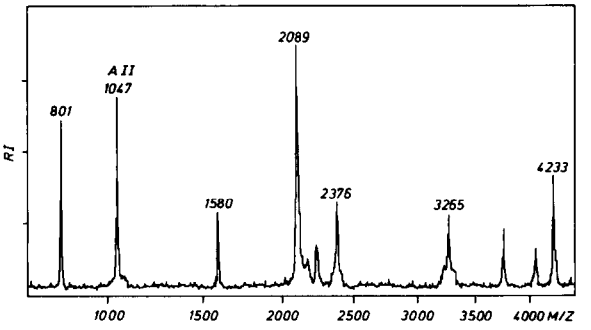

Fig. 5. MALDI mass spectrum of the bioactive fraction obtained after RP-HPLC (Fig. 4). AII indicates a signal of a protonated ion, which is identical with the mass of the protonated ion of angiotensin II.

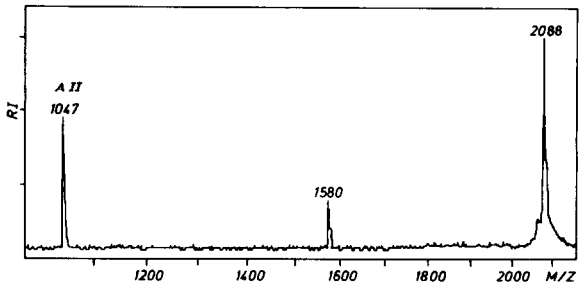

Fig. 7. MALDI mass spectrum of the desalted bioactive fraction from cation-exchange chromatography (Fig. 6). AII indicates a signal of a protonated ion which is identical with the mass of the protonated ion of angiotensin II.

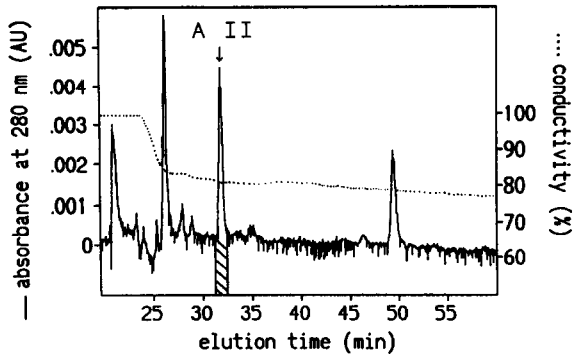

Fig. 8. Micropreparative RP-HPLC of the bioactive fraction of the cation-exchange chromatography (Fig. 6). Absorbance range, 0.1. Conditions: column, C_{18} GROM-SIL 300-ODS-2 (100 × 2 mm I.D.); eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile; gradient, 0–14 min 100% eluent A, 14–20 min 0–22% eluent B, 20–70 min 22–27% eluent B; flow-rate, 100 μl/min; sample, bioactive fraction from the semi-preparative chromatography (Fig. 6; 300 ml plasma equivalent) dissolved in 1 ml of eluent A; fraction size, 200 μl. The vasoactive fraction, equivalent to 1 ml of plasma, caused an elevation of perfusion pressure of 6 mmHg (hatched area) in the bioassay. AII indicates the retention time of angiotensin II.

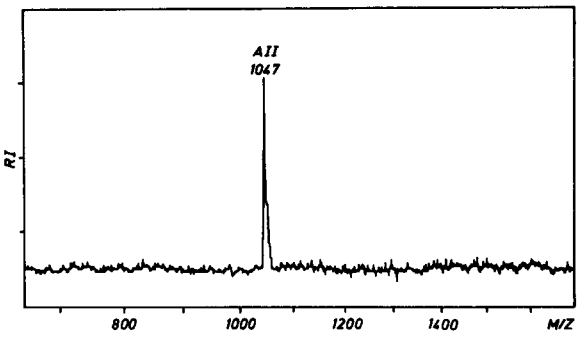

Fig. 9. MALDI mass spectrum of the bioactive fraction obtained after RP-HPLC (Fig. 8). AII indicates a signal of a protonated ion which is identical with the mass of the protonated ion of angiotensin II.

perfused rat kidney before and after incubating the kidney with the angiotensin receptor antagonist saralasin. The action of the fraction was completely blocked by saralasin (Fig. 3).

Determination of the recovery of angiotensin II liberated by the proteolysis

Table II shows that the amount of angiotensin II produced under the experimental conditions described above is about 1000 times higher than the amount of free angiotensin II in plasma.

DISCUSSION

Cross-flow filtration is not very commonly used as an alternative to dialysis or pressure ultrafiltration. In the literature on protein purification [2,17–20] cross-flow filtration has received little interest compared with dialysis, pressure ultrafiltration and ultrafiltration by centrifugation. One of the reasons may be that it was originally designed for biotechnological filtration and therefore it is felt to be too crude to handle small-scale amounts. Our results show that cross-flow filtration adjusted to laboratory-scale volumes has several advantages. First, the major benefit of cross-flow filtration is that even crude liquids can be filtered, such as plasma with submandibular gland extract, because the construction of the cross-flow filtration prevents membrane clogging [21]. Second, cross-flow filtration compared with dialysis shows better results in retaining molecules larger than the labelled retention limit

TABLE II

AMOUNTS OF ANGIOTENSIN II IN PLASMA, ANGIOTENSINOGEN AND THE BIOACTIVE FRACTION OF THE SEMI-PREPARATIVE RP-HPLC (FIG. 4)

Angiotensin II	Concentration
Free angiotensin II in plasma [15]	4–26 pg/ml
Total angiotensin II released from angiotensinogen [16]	10–55 ng/ml
Angiotensin II released by incubation of plasma with submandibular gland extract[a]	25 ± 5 ng/ml

[a] Mean ± S.D. (n = 5) calculated from the dose–response curve (Fig. 2).

(Fig. 1). Third, as one or more pretreatment steps may become unnecessary, the recovery rate should be higher. Fourth, cross-flow filtration is ideal prior to chromatography, because the filtrate can be easily chromatographed and the column following the filtration step is protected, because the filtration removes particles. Fifth, cross-flow filtration is not only useful for the separation of peptides, or other biologically molecules, from the reaction mixture but also allows the reaction to be controlled. In contrast to the conventional dialysis technique or pressure ultrafiltration, the retentate and filtrate can be monitored (*i.e.*, pH) and manipulated continuously and independently of each other during the reaction. For example, as was shown above, the retentate and filtrate can be kept at different temperatures.

Following the cross-flow filtration step the peptides had to be concentrated from a large volume of buffer (1 l). For this RP-SPE, which had been shown by many investigators to be a fast and reproducible method with high recovery rates [17,22–24], was chosen and modified to fit our needs. RP-SPE was not performed on ready-to-use cartridges but the RP material was packed into silanized glass tubes with PTFE frits. This procedure has the advantage that several grams of sorbent can be packed in one column. Also, the elution of interfering materials from the frits and polymer column material is significantly reduced [25]. This is important in bioassays and in structural investigations of the bioactive fractions.

Next, the biologically active peptides, usually present in trace amounts, had to be isolated from a complex substance mixture. Thus, specific and sensitive detection methods and an optimum purification procedure were required.

The requirement for specific detection was accomplished with the bioassay. The detection limit of this bioassay is in the range 0.1–1 ng of angiotensin II (total amount injected into the kidney perfusion system, Fig. 2). The control of the purity of the fractions and the identification of the vasoactive peptide were possible with the help of the sensitive method of MALDI-MS [14] (Figs. 5, 7 and 9).

With regard to an optimum purification procedure, the benefits of the first two sample preparation steps, cross-flow filtration and RP-SPE, are mentioned above. After this, the peptide-rich fraction was further purified with three chromatographic steps, preparative RP-HPLC (Fig. 4), followed by microbore cation-exchange HPLC (Fig. 6) and finally by microbore RP-HPLC (Fig. 8), giving a homogeneous biologically active peptide fraction. The homogeneity of the purest fraction was confirmed by MALDI-MS, shown in Fig. 9. The particular benefit of the chromatographic steps is the use of micropreparative HPLC, which is optimized to handle low-μg–ng amounts.

The active peptide was clearly identified as angiotensin II. The identity was proved by MALDI-MS (Fig. 9), by comparison of the retention times of the active fraction and angiotensin II, which appeared to be very similar (Figs. 4, 6 and 8), and by specific pharmacological inhibition (Fig. 3). The action of the vasoactive fraction was blocked by the angiotensin II receptor antagonist saralasin.

The determination of the recovery of angiotensin II, liberated by proteolysis, demonstrates that after incubation of plasma with an extract, rich in proteases, bioactive peptides can be released. The total recovery of released angiotensin II can be roughly estimated to be more than 50% of total releasable angiotensin II.

The biochemical aspects of the results are due to the fact that the submandibular gland contains tonin [6] and several tonin-like serine proteases [7], which may be responsible for the liberation of angiotensin II. Tonin is known to cleave angiotensin II directly from angiotensinogen [5]. Further, it should be emphasized that the isolation and determination of angiotensin II was not the primary aim of the study, although the analyses finally revealed angiotensin II as the reaction product.

CONCLUSION

A method based on cross-flow filtration, which is suitable for the isolation of bioactive peptides released by proteases, has been developed. The method is attractive for isolating peptides released by proteases from any biological material. It should be possible to extend the use of the benefits of the cross-flow filtration not only to the study of proteolysis and the reaction products but also to other applications dealing with reactions of biological systems.

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