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Principle and applications of the protein-purification-parameter screening system

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Abstract

For the purification of a target protein, liquid chromatography is the method of choice, if its activity has to be maintained. The selection of optimum parameters will improve in proportion to the number of individual parameters varied in initial experiments. Here a fast screening method is described, which utilizes automated parallel chromatographic experiments in the batch mode in 96-well plates. The principle of this protein-purification-parameter screening (PPS) system is demonstrated with a mixture of four proteins. An application of PPS for the determination of a purification step of an angiotensin-II-generating enzyme from a crude tissue extract is shown.

Keywords: Protein-purification-parameter screening; Proteins

1. Introduction

For the purification of proteins liquid chromatography is preferred if the activity of a target protein has to be maintained. Usually the aim of a protein-purification process is the removal of accompanying biomolecules from a target protein. Unfortunately every purification step is associated with partially loss of the target protein. The amount of this loss is depending on unspecific interactions of the target protein with the matrix of the stationary phase and the degree of denaturation, precipitation or irreversible binding. Thus a purification strategy of a target protein should involve as few steps as possible. This aim can be achieved, if the individual chromatographic steps are as specific as possible. It is desired to achieve a high specific yield together with a high recovery by each purification step [1,2].

The resolution of liquid chromatography depends on parameters such as pH or ionic strength, the sample composition as well as additives. These additives are necessary to stabilize the target protein [3] and the chromatographic medium, including functional groups and the matrix [4]. The number of individual parameters, which have to be varied to find an optimal purification step, depend on the kind of chromatography and the properties of the target protein. Systematic variations of all relevant parameters will help to observe the optimal parameters for a chromatographic purification step. The number of convenient experiments to find out the optimum parameters can quickly exceed 100 individual experiments [5]. Therefore automation of these experiments will help to speed up finding appropriate chromatographic parameters for the purification of a protein.

Three commercial HPLC systems are available for the automated search for chromatographic parameters of proteins. The systems offered by Amersham Bioscience (e.g. Äkta Explorer, http://www4.amershambiosciences.com) [6], Applied Biosystems (BioCAD 700E Workstation, http://www.appliedbiosystems.com) and Bio-Rad (Bio-Logic DuoFlow, http://www.bio-rad.com) allow the varying of sample-application buffers and elution buffers, gradients and pre-packed columns. All three systems perform the experiments in series varying one parameter per chromatographic run. Thus the variation of a larger number of parameters is time consuming. Furthermore, to these HPLC systems only samples can be applied, which are free of particles and as a result do not block the chromatography

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system. Normally raw extracts containing particles cannot be analyzed with these HPLC systems.

In order to allow the systematic and fast search for appropriate chromatographic parameters for the purification of a target protein, we developed a parallel protein-purification-parameter screening (PPS) system based on batch chromatography in deep-well plates in the 96-well format, with which even raw extracts can be processed. In this study the principle and the performance of the protein-purification-parameter screening system is described. The principle of the system is demonstrated with a mixture of proteins (ribonuclease A, cytochrome *c*, lysozyme and myoglobin), in this article referred as standard proteins. As an application the search for parameters for a chromatographic purification step of an angiotensin-II-generating enzyme from a raw extract from renal tissue is described. Furthermore the application of parameters determined with the PPS is demonstrated for diverse chromatographic separations.

2. Experimental

2.1. Materials

Sodium chloride, disodium hydrogenphosphate dihydrate, potassium phosphate, sodium phosphate, sodium chloride, Fractogel EMD SO_3^- (M) and Fractogel EMD TMAE HiCap(M) were bought from VWR International (Darmstadt, Germany). Acetic acid, sodium acetate, formic acid, malonic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES) and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), cytochrome *c*, lysozyme, myoglobin and ribonuclease A were from Sigma–Aldrich (Taufkirchen, Germany). Sodium hydrogencarbonate was purchased from AppliChem (Darmstadt, Germany). Sodium acetate was from Serva (Heidelberg, Germany). MacroPrep DEAE Support was bought from Bio-Rad (Munich, Germany). DEAE Sepharose FastFlow and DEAE Sephacel were purchased from Amersham Biosciences (Freiburg, Germany). Porcine kidneys were received for local slaughterhouse.

2.2. Instrumentation

The PPS experiments were performed in 2.2 ml deep-well plates from Abgene (Hamburg, Germany). The robot system Multiprobe-II-ex from Perkin-Elmer (Boston, USA) was used for liquid handling steps.

For gradient chromatography we used the ÄKTA Explorer System from Amersham Biosciences (Uppsala, Sweden).

Cation-exchange chromatography was performed on HR10/2 columns from Amersham Biosciences (Uppsala, Sweden), which were filled with 2 ml of Fractogel EMD SO_3^- (M), from Merck (Darmstadt, Germany). For the quantification of the individual standard proteins the HPLC system SMART from Amersham Bioscience (Uppsala, Sweden) with the reversed-phase column TSKgel Super-Octyl (5.0 cm × 4.6 mm i.d.) from Tosoh Bioseparation (Stuttgart, Germany) was used.

Two-dimensional electrophoresis (2-DE) was performed employing own equipment from WITA (Teltow, Germany). Silver-stained 2-DE gels were scanned using a ScanJet 6300C equipment (Hewlett-Packard, Palo Alto) at 1200 dpi. Raw 2-DE images were analyzed using the Melanie III software (GeneBio, Geneva, Switzerland).

2.3. Methods

2.3.1. Measurement of protein concentration

Ten microliters sample was mixed with 290 μl of Coomassie reagent in a microtiter plate and the UV absorption was measured with a UV-reader system. The protein concentration was calculated from the UV absorption and an albumin concentration curve.

2.3.2. PPS experiments with standard proteins

2.3.2.1. PPS for cation-exchange chromatography. For the PPS experiments, deep-well plates containing identical compositions of the sample-application buffers were prepared according to Table 1. Each buffer (40 mmol/l) was adjusted to the pH given in brackets: citric acid (pH 3), formic acid (pH 4), acetic acid (pH 5) malonic acid (pH 6), MES (pH 6.5), phosphoric acid (pH 7), phosphoric acid (pH 7.5) and HEPES (pH 8). The buffers (1 ml/well) were transferred to the wells of the 96-well plate according to Table 1. Each well contained 1 ml buffer. To each well NaCl, dissolved in water in individual concentrations, according to Table 1, was added (final volume 2 ml).

For the cation-exchange chromatography PPS experiments 100 μl of Fractogel EMD SO_3^- (M) gel was filled in each of 32 wells (8 × 4 matrix) of a 96-deep-well plate. Each gel was washed three times with 460 μl of the individual buffers by copying the buffers from the sample-application-buffer deep-well plates to the deep-well plate filled with gel. After equilibration to each of the gels

Table 1
Scheme of the sample-application-buffer deep-well plate used for the cation-exchange screening experiments

NaCl				pH
0 mM	100 mM	200 mM	500 mM	
A 1	A 2	A 3	A 4	3
B 1	B 2	B 3	B 4	4
C 1	C 2	C 3	C 4	5
D 1	D 2	D 3	D 4	6
E 1	E 2	E 3	E 4	6.5
F 1	F 2	F 3	F 4	7
G 1	G 2	G 3	G 4	7.5
H 1	H 2	H 3	H 4	8

"D 3" for example represents a buffer with a pH of 6 and a sodium chloride concentration of 200 mM.

1.5 ml of the sample-application-buffer from the deep-well buffer plate was copied.

The sample of standard proteins consisted of ribonuclease A, cytochrome *c*, lysozyme and myoglobin (40 mg each), dissolved in 6.4 ml water. Two hundred microliters aliquots of the protein mixture were added to the 32 wells of the deep-well plate, filled with the equilibrated gels and the sample-application buffers. Samples, buffers and the gels were carefully mixed. After sedimentation of the gels (about 2 min) the supernatants were removed and the gels were washed three times by re-applying 460 μl of the individual buffers per well from the sample-application-buffer deep-well plate to the deep-well gel plate. The washing procedure included the addition of the individual buffers to the corresponding gels, mixing of buffers and gels and removing of the supernatants from the gels after sedimentation of the gels. The proteins were eluted from the gels by adding 100 μl of 2 M aqueous NaCl, and suspending the elution-buffer with the gel. After sedimentation of the gel the eluate (supernatant) was transferred to an empty deep-well plate. The elution step was repeated twice and the eluates pooled in the individual wells of the deep-well plate. The eluates were analyzed by reversed-phase HPLC (see Section 2.3.2.2).

2.3.2.2. Quantification of the standard proteins in the eluates of the deep-well batch chromatography. The eluates from the PPS experiment (Section 2.3.2.1) were analyzed with reversed-phase chromatography. The mobile phase was composed of 0.05% trifluoroacetic acid (TFA) in HPLC-grade water (solvent A) and 0.05% TFA in acetonitrile (solvent B). Twenty microliters of the eluates of the 96-well batch chromatography (Section 2.3.2.1) were mixed with solvent A (100 μl final volume; case A). Nine hundred microliters from each fraction from the gradient chromatographies (Section 2.3.2.3) were mixed with 100 μl 1% TFA in distilled water (1000 μl final volume; case B). The chromatography system employed a reversed-phase column TSKgel Super-Octyl, a flow-rate of 1 ml/min, 100 μl sample loop (case A) or 1000 μl sample loop (case B), 13.3 min run time from 23% of solvent B to 42% of solvent B, and a spectrophotometric detector at 214 nm. For the quantification of the individual standard proteins the areas under the curves were integrated and compared with the individual calibration curves of the standard proteins.

2.3.2.3. Gradient chromatography. For each chromatography a sample consisting of 2.5 mg each of ribonuclease A, cytochrome *c*, lysozyme and myoglobin was used. For all experiments the chromatography system employed a flow-rate of 2 ml/min and was equipped with a 1000 μl sample loop and a spectrophotometric detector at 280 nm and 405 nm.

Cation-exchange chromatography at pH 3. Forty micromoles citric acid, pH of 3, was used as solvent A. Solvent B additionally contained 2 M sodium chloride. The sample was dissolved in 1 ml solvent A. For the gradient chromatography a gradient with 90 min run time from 0% of solvent B to 75% of solvent B was used.

Table 2
Scheme of the sample-application-buffer deep-well plate used for the anion-exchange screening experiments

NaCl				pH
0 mM	100 mM	200 mM	500 mM	
A 1	A 2	A 3	A 4	5
B 1	B 2	B 3	B 4	6
C 1	C 2	C 3	C 4	7
D 1	D 2	D 3	D 4	7.5
E 1	E 2	E 3	E 4	8
F 1	F 2	F 3	F 4	8.5
G 1	G 2	G 3	G 4	9
H 1	H 2	H 3	H 4	10

"F 2" for example represents a buffer with a pH of 8.5 and a sodium chloride concentration of 100 mM.

Cation-exchange chromatography at pH 6. For the cation-exchange chromatography at pH 6 malonic acid (40 mM, solvent A) was used as buffer. Solvent B was prepared by adding 1M NaCl to solvent A. The sample was dissolved in 1 ml solvent A. The gradient was developed within 60 min from 0% solvent B to 100% solvent B.

2.3.2.4. PPS experiments with anion-exchange gels. The anion-exchange chromatography screening experiments were performed with sample-application buffers listed in Table 2. The following buffers were used (pH values are given in brackets): *N*-methylpiperazine (pH 5), Bis–Tris (pH 6), Bis–Tris–propane (pH 7), triethanolamine (pH 7.5), Tris (pH 8), diethanolamine (pH 8.5), ethanolamine (pH 9) and 1,3-diaminopropane (pH 10). To each of the wells individual amounts of NaCl were added according to Table 2.

Four different anion-exchange gels, Fractogel EMD TMAE HiCap, DEAE Sepharose Fast-Flow, Macro-Prep DEAE Support and DEAE-Sephacel were transferred into four deep-well plates. In each of the wells 100 μl gel was applied. For the anion-exchange chromatography experiments a 4 × 8 matrix was used, parallel to the sample-application-buffer matrix (Table 2).

For the equilibration each gel was washed three times with 460 μl buffer copied from the sample-application-buffer plate (given in Table 2). After equilibration, buffers copied from the sample-application-buffer plate were added to the gels (800 μl buffer/well).

Albumin dissolved in water (3 mg/ml) was used as sample. Hundred microliters aliquots of the sample were applied to the gels in the 32 wells of the four different plates. The gels were mixed carefully with the samples. After sedimentation of the gels (about 2 min) the supernatants were removed and the gels were washed with buffer copied from the sample-application-buffer plate (460 μl individual buffer per well).

After washing the gels the absorbed proteins were eluted by adding 100 μl 2 M NaCl (8.4 pH) to every well, suspending the elution-buffer with the gel and by removing the supernatant from the gel, after sedimentation of the gel. The elution step was repeated twice. All three eluates were pooled. The eluates were collected in deep-well plates. The protein concentration of the eluates were analyzed with a Bradford assay (Section 2.3.1).

2.3.3. Chromatography of porcine renal proteins

2.3.3.1. Preparation of the protein extract from porcine renal tissue. Porcine renal tissue was collected at the local slaughterhouse. Immediately after excision the tissue was cut into pieces of 1 ml and frozen in liquid nitrogen. The pieces were dried in vacuum and thereafter pulverized. One gram of the powder was homogenized in 15 ml 10 mM phosphate buffer (pH 7.3). The homogenate was centrifuged for 30 min at 30 000 × *g* and 4 °C (Sorvall SS-34 rotor), twice. The protein concentration of the supernatant was adjusted to 10 mg/ml with 10 mM phosphate buffer (pH 7.3).

2.3.3.2. PPS experiments for the determination of parameters for the purification of an angiotensin-II-generating enzyme from the renal protein extract with cation-exchange chromatography. Aliquots of 200 μl of the renal protein extract (protein concentration: 10 mg/ml) were applied to the mixtures of gels (300 μl/well) and sample-application buffers (1500 μl/well). After sample-application the gels were washed (3×) with buffers copied from the sample-application-buffer plate (600 μl/well of the individual buffers). The absorbed proteins were eluted with the addition (3×) of 2 M NaCl (300 μl/well). The supernatant of all wells were analyzed for an angiotensin-II-generating activity as described in [7] and the protein concentration of each fraction was measured.

2.3.3.3. Sample-displacement cation-exchange chromatography of a crude porcine renal protein extract. For the first chromatographic purification step of an angiotensin-II-generating enzyme from a porcine renal protein extract parameters, determined in Section 2.3.3.2, were used.

Twelve microliters cation-exchange gel (Fractogel EMD SO_3^-) was washed with distilled water, first, followed by 40 mM citric acid (pH 3) containing 500 mM NaCl. The gel was distributed to 12 × 15 ml tubes.

A porcine kidney was prepared as described in Section 2.3.3.1. Five grams porcine renal tissue powder was homogenized in 50 ml of 20 mM phosphoric buffer (pH 7). After homogenization 2.1 g proteins were dissolved in the buffer. To the protein extract 40 mM citric acid and 500 mM NaCl were added and the mixture was adjusted to pH 3. After centrifugation the supernatant of the renal protein extract (150 mg total protein) was mixed with 1 ml gel. After sedimentation of the gel the supernatant was transferred to a second tube (tube number 2) containing 1 ml gel. This procedure was repeated 10 times. Each of the individual gels (in the tubes number 1–12) were washed three times with 40 mM citric acid buffer (pH 3) containing 500 mM NaCl. For elution of the absorbed proteins the gels from each tube were mixed with 1 ml 100 mM sodium hydrogen carbonate (pH 8.3) with 2 M NaCl added. After sedimentation of the gels the supernatants were transferred to 12 tubes. The elution was repeated twice. The angiotensin-II-generating activity of the eluates was measured by the MES method as described in [7].

2.3.3.4. Gel electrophoresis and image analysis. High-resolution 2-DE was performed by combining isoelectric focusing (IEF, first dimension) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, second dimension), as described by Klose and Kobalz [8] with some alterations. IEF was performed in rod gels (inner diameter for the analytical gels 0.09 cm and for the preparative gel 0.15 cm) containing 3.5% acrylamide, 0.3% piperazine diacrylamide and 4% (w/v) carrier ampholytes. Renal proteins (50 μg) were loaded per rod (1.5 mm thickness) and separated according to the WITA protocols [9]. Gels were stained with the MS-compatible silver staining procedure [10]. Bands were automatically detected without previous contrast enhancement, followed by manual edition when necessary.

3. Results and discussion

3.1. Chromatography of standard proteins

To demonstrate the principle of the PPS, aliquots of a mixture containing cytochrome *c*, lysozyme, myoglobin and ribonuclease A (standard proteins) were applied to equal volumes of a cation-exchange gel, which was present in 32 wells of a deep-well plate. The method underlying the PPS involves four steps: (1) The stationary phase must be equilibrated to individual buffers from the sample-application-buffer plate. (2) After equilibration aliquots of the sample can be applied to the gels. (3) Non-binding biomolecules will be removed by washing steps. (4) The absorbed proteins are eluted with an appropriate elution buffer. The steps 1–4 include (a) mixing the gels in the deep-well plates with (sample-application- or elution-) buffers copied from the (sample-application- or elution-) buffer plates or the sample aliquots, (b) waiting for the sedimentation of the gels, and (c) removing the supernatant from the gel after sedimentation.

In this study prior to sample-application the gels in the 32 individual wells were equilibrated to individual sample-application buffers listed in Table 1 (for the cation-exchange screening experiments) and in Table 2 (for the anion-exchange screening experiments). After sample-application, washing and elution steps, the compositions of the eluates of the 32 individual gels were

Table 3
Absolute yields (in %, 100% is equal to the originally applied amount of each protein) of ribonuclease A, cytochrome *c*, lysozyme and myoglobin (referred as standard proteins) of a 32-well PPS experiment with cation-exchange chromatography

NaCl (mM)	pH							
	3	4	5	6	6.5	7	7.5	8
Ribonuclease A								
0	29	37	36	42	33	3	19	35
100	28	36	31	24	25	5	1	3
200	24	30	28	6	4	0	0	0
500	16	4	0	0	0	0	0	0
Lysozyme								
0	27	70	43	60	59	52	51	52
100	31	63	52	55	55	52	43	54
200	45	38	45	30	36	22	0	0
500	29	5	0	0	0	0	0	0
Cytochrome *c*								
0	31	51	51	74	68	44	64	71
100	43	56	51	38	45	30	19	57
200	42	48	28	4	6	3	0	0
500	40	10	0	0	0	0	0	0
Myoglobin								
0	5	16	12	0	0	0	0	0
100	6	12	7	0	0	0	0	0
200	4	10	2	0	0	0	0	0
500	2	12	0	0	0	0	0	0

Aliquots of the sample (1.25 mg of each protein) were applied to 100 μl cation-exchange gel per well equilibrated with copies from the sample-application-buffer plate (Table 1). After washing the gel with the corresponding buffers from the sample-application-buffer plate, the absorbed proteins were eluted with 3 × 100 μl 2 M aqueous NaCl.

analyzed by reversed-phase HPLC. Table 3 summarizes the results of the PPS experiment, a parallel 32-well batch chromatography, and gives the yields of each protein in percent. As expected, the highest yields of the individual proteins were obtained in the absence of NaCl. Binding of the individual proteins depend on their isoelectric points and therefore from the pH of the buffers. The highest yields for lysozyme and myoglobin were obtained at pH 4 and for cytochrome *c* and ribonuclease A at pH 6. These results can be used to achieve a high absolute recovery of a target protein independently from its purity. If high yields *and* high purities are desired, the calculation of specific yields is more helpful (Table 4). The pattern of the highest specific yields is significantly different from the pattern of the absolute yields demonstrated in Table 3. For each individual protein individual pairs of parameters (pH and ionic strength) with maximum specific yields can be recognized. The highest specific yield for lysozyme is obtained with the cation-exchange gel at pH 7 in the presence of 200 mM NaCl. Using these conditions ribonuclease A and myoglobin do not bind to the cation-exchange gel. Only small amounts of cytochrome *c* are absorbed. The adsorption specificity of lysozyme may be explained not only by electrostatic but also by other interactions, e.g. hydrophobic interactions, which increase with increasing salt concentrations, may be

Table 4
Specific yields in percentage (100% is equivalent to pure protein) of ribonuclease A, cytochrome *c*, lysozyme and myoglobin of the 32-well PPS experiment, calculated from the absolute yields given in Table 3

NaCl (mM)	pH							
	3	4	5	6	6.5	7	7.5	8
Ribonuclease A								
0	32	21	25	24	21	3	14	22
100	26	22	22	21	20	6	2	3
200	21	24	27	15	9	0	0	0
500	18	13	0	0	0	0	0	0
Lysozyme								
0	29	40	30	34	37	53	38	33
100	29	38	37	47	44	60	68	47
200	39	30	44	75	78	88	0	0
500	33	16	0	0	0	0	0	0
Cytochrome *c*								
0	34	29	36	42	43	44	48	45
100	40	34	36	32	36	34	30	50
200	37	38	27	10	13	12	0	0
500	46	32	0	0	0	0	0	0
Myoglobin								
0	5	9	8	0	0	0	0	0
100	6	7	5	0	0	0	0	0
200	3	8	2	0	0	0	0	0
500	2	39	0	0	0	0	0	0

causal for the high specific yields of cytochrome *c* at pH 3 and of myoglobin at pH 4 (Table 4).

How can the data, obtained in the PPS experiments, be used for the purification of a target protein with column chromatography? The answer to this question depends on the aim of the protein purifier: (I) If a high absolute yield irrespective from its purity is needed, the PPS data can be searched for the highest absolute yield. (II) If a high purity of the target protein is desired, the specific yield of the target protein should be calculated from the PPS data, e.g. the highest specific yield of ribonuclease A in the batch chromatographic PPS experiment was obtained at pH 3 in the absence of NaCl (Table 4). For the cation-exchange gradient chromatography, this pair of parameters was used for eluent A, the equilibration and sample-application buffer. The mixture of standard proteins was dissolved in this buffer and after injection of the sample a gradient was run. The resulting chromatogram (Fig. 1) shows two nearly base-line separated peaks eluting within the gradient. Analysis of both fractions revealed that in the first fraction ribonuclease A and lysozyme coeluted, whereas in the second fraction nearly homogenous cytochrome *c* was detected. Compared to the PPS experiment the specific yield of the gradient chromatography of ribonuclease A increased from 32% to 49%. This effect is caused by the separation of cytochrome *c* from the ribonuclease A.

In the second example the results from the PPS experiment were used to purify myoglobin by a cation-exchange frontal chromatography. pH 6 in the absence of NaCl was

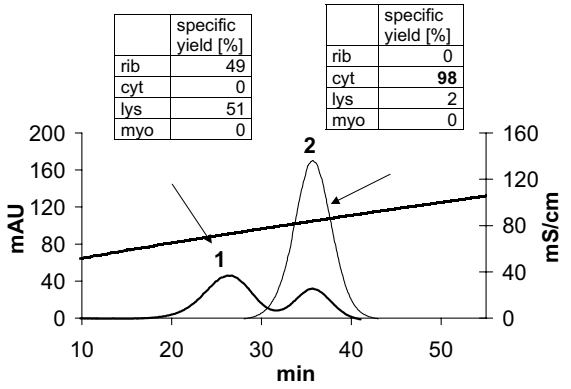

Fig. 1. Chromatogram of a cation-exchange gradient chromatography of a mixture of ribonuclease A, cytochrome *c*, lysozyme and myoglobin (each 2.5 mg) using a pair of parameters (pH 3, 0 mM NaCl) from the PPS experiment (Table 4). Column: 2 ml Fractogel EMD SO_3^- packed in a HR 10/2 column. Detection of UV absorption was performed at 280 nm (thick black line) and 405 nm (thin black line). Conductivity was measured (black thick line). The inserted tables show the specific yields of every protein in the fractions labeled with 1 and 2. The mobile phase was composed of 40 mM citric acid (pH 3) (eluent A). Eluent B consisted of eluent A with 2 M NaCl added. The sample was dissolved in eluent A. The chromatography system employed a flow-rate of 2 ml/min, 1000 μl injection volume and 90 min run time from 0% of eluent B to 75% of eluent B.

chosen as equilibration and sample-application buffer (eluent A), because myoglobin was not retained in contrast to all other proteins, which absorbed with high affinities using these conditions (Table 3). The behavior of the standard proteins, observed with the PPS experiment, is reflected by the cation-exchange column chromatography (Fig. 2). Myoglobin elutes directly behind the void volume, without being contaminated with any other protein. All other proteins desorbed from the gel within the gradient.

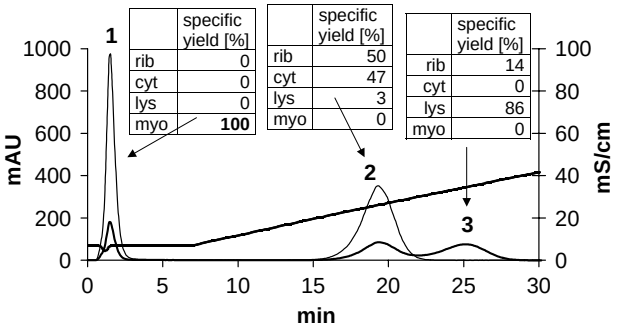

Fig. 2. Chromatogram of a cation-exchange gradient chromatography of a mixture of ribonuclease A, cytochrome *c*, lysozyme and myoglobin (each 2.5 mg) using a pair of parameters (pH 6, 0 mM NaCl) from the PPS experiment (Table 3). The inserted tables show the specific yields of every protein in the fractions labeled 1, 2 and 3. The mobile phase was composed of 40 mM malonic acid (pH 6) (eluent A) and 40 mM malonic acid (pH 6) with 1 M NaCl (eluent B). The sample was dissolved in eluent A. The chromatography system employed a flow-rate of 2 ml/min, 1000 μl injection volume and 60 min run time from 0% of eluent B to 100% of eluent B. Further details are given in the legend of Fig. 1.

Figs. 1 and 2 demonstrate a limitation of the PPS approach. With the PPS data it can be predicted if a target protein bind to a stationary phase and if the protein can be desorbed from the stationary phase, retaining its activity, but it cannot be predicted if and how individual components of a mixture are separated from each other within the gradient. This question must be clarified with additional experiments, which should also include searching for the optimum shape of the gradient. For this purpose the commercially available HPLC-based parameter-scouting systems are recommended.

In a further PPS experiment not only the buffers were varied but also the stationary phase. Four different anion-exchange gels were investigated towards their properties to bind albumin (Table 5). The highest yields range from 302 μg (Fractogel EMD TMAE; pH 10, 0 mM NaCl) to 154 μg (DEAE Sephacel, pH 10, 100 mM NaCl). The highest yields are obtained at pH 9 and 10 (Fractogel EMD TMAE; DEAE Sepharose; DEAE Sephacel). In contrast to these results, the highest yield of the eluates of the Macro-Prep DEAE gel was measured at pH 6, pointing to different interactions of albumin with the different anion-exchange gels depending on pH and NaCl concentrations. These differences are also very prominent at pH 10 in the absence of

Table 5
Absolute yields of albumin of a 4× 32-well anion-exchange PPS experiment, varying sample-application buffers and anion-exchange gels

NaCl (mM)	pH							
	5	6	7	7.5	8	8.5	9	10
Fractogel EMD TMAE HiCap								
0	25	261	276	276	263	267	293	302
100	4	109	134	216	238	267	268	271
200	2	4	5	19	4	5	9	9
500	4	2	3	0	4	4	4	3
MacroPrep DEAE Support								
0	11	230	211	212	219	212	137	5
100	3	84	106	157	169	104	28	3
200	1	12	22	53	37	16	6	4
500	3	3	5	2	0	7	4	5
DEAE Sepharose FastFlow								
0	34	36	222	222	226	229	238	231
100	1	10	62	140	168	177	144	78
200	2	5	4	3	3	3	5	4
500	0	3	3	2	3	2	5	3
DEAE Sephacel								
0	4	137	148	136	137	132	152	151
100	0	53	63	112	131	146	153	154
200	2	2	2	7	19	35	41	76
500	1	3	2	1	1	2	4	3

The albumin concentration is given in micrograms. One hundred microliters gel per well was used. Prior to the sample application the gels were equilibrated with buffers, copied from the sample-application-buffer plate (Table 2). Three hundred micrograms albumin was applied to each well. After washing the gel with the corresponding buffers from the sample-application-buffer plate (Table 2), the absorbed proteins were eluted with 3 × 100 μl of 2 M aqueous NaCl.

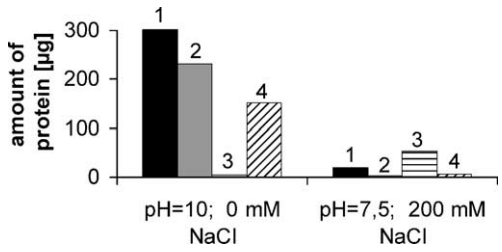

Fig. 3. Details of the results of the PPS experiment of Table 5. (1) Fractogel EMD TMAE HiCap, (2) DEAE Sepharose FastFlow, (3) Macro Prep DEAE Support, (4) DEAE Sephacel.

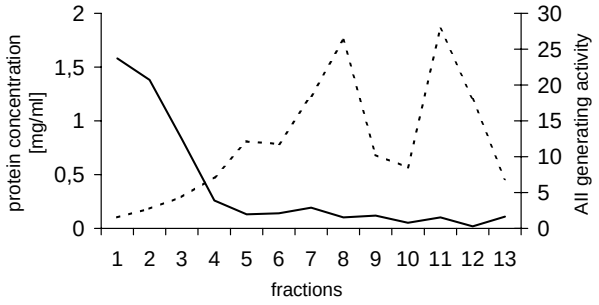

Fig. 4. Chromatogram of a cation-exchange sample-displacement chromatography for the purification of an angiotensin-II-generating enzyme from a porcine renal protein extract, using parameters determined with a PPS experiment (Table 6). The solid line represents the protein concentration, the dotted line the angiotensin-II-generating activity. Cation-exchange gel: Fractogel EMD SO_3^- (1 ml/well).

NaCl and pH 7.5 in the presence of 200 mM NaCl (Fig. 3). At pH 10 MacroPrep DEAE binds only 2% of the amount of albumin as Fractogel EMD TMAE. In contrast at pH 7.5 in the presence of 200 mM NaCl Fractogel EMD TMAE binds only 6% of the amount of albumin compared to MacroPrep DEAE. This effect may be caused by hydrophobic interactions, which become prominent with increasing NaCl concentrations.

In the first part of this study, the principle of the PPS was demonstrated by using standard proteins. In the second part the question was followed if PPS can be applied to complex protein fractions containing hundreds of different proteins. For this purpose a homogenate of porcine renal tissue was chosen. The homogenate was applied to cation-exchange gels equilibrated to different pH in the presence of varying concentrations of NaCl (Table 6). Protein concentrations and angiotensin-II-generating activities were measured and the specific enzymatic activity calculated. The largest amount of proteins was adsorbed at pH 6. The highest yield of enzymatic activity and specific activity was measured in the eluate of the PPS chromatography with pH 3 and 500 mM NaCl added. These conditions, unusual for ion-exchange chromatography, indicate the presence of secondary interactions, different from the electrostatic (primary) interactions. This pair of parameters was used for the purification of an angiotensin-II-generating enzyme. A preparative purification in the sample-displacement mode was performed, using an equilibration- and sample-application buffer with pH 3, with 500 mM NaCl added. The chromatogram (Fig. 4) shows two different fractions with angiotensin-II-generating activities, which both elute separately from the fractions with high UV absorbance. The enzymatic active fractions 11 + 12 were analyzed by 2-DE (Fig. 5B). The pattern of proteins is completely different compared to the pattern of the renal protein extract (Fig. 5A), the sample, which was applied to the cation-exchange sample-displacement chromatography. The effective enrichment of acidic proteins, which were not recognizable on the 2-DE of the homogenate, is due to the separation mechanisms of the cation-exchange sample-displacement chromatography. The discrimination of a huge set of proteins is a result of the

Table 6
PPS experiment for the determination of parameters for the purification of an angiotensin-II-generating enzyme from a porcine renal protein extract by cation-exchange chromatography

pH	NaCl (mM)											
	Protein concentration				Enzyme activity				Specific activity			
	0	100	200	500	0	100	200	500	0	100	200	500
3	36	26	28	22	54	93	102	162	2	4	4	7
4	49	54	51	32	8	15	16	14	0	0	0	0
5	60	51	41	9	20	11	42	8	0	0	1	1
6	66	47	20	5	12	14	22	12	0	0	1	2
6.5	57	38	16	6	10	21	18	17	0	1	1	3
7	38	19	12	5	31	10	64	14	1	1	5	3
7.5	20	17	11	5	8	11	20	23	0	1	2	5
8	26	15	10	4	30	18	24	28	1	1	2	7

For the PPS experiment a cation-exchange gel (300 µl) was used. Aliquots of the sample (2 mg proteins) were applied each to the individual gels, equilibrated with the sample-application buffers specified in Table 6. After washing the gel with the corresponding buffers, the absorbed proteins were eluted with the elution buffer. Enzymatic activity was determined with the MES system [7]. The left table summarizes the protein concentrations of the individual eluates. The table in the middle gives the angiotensin-II-generating enzyme activity. In the right table the individual specific enzyme activities are calculated.

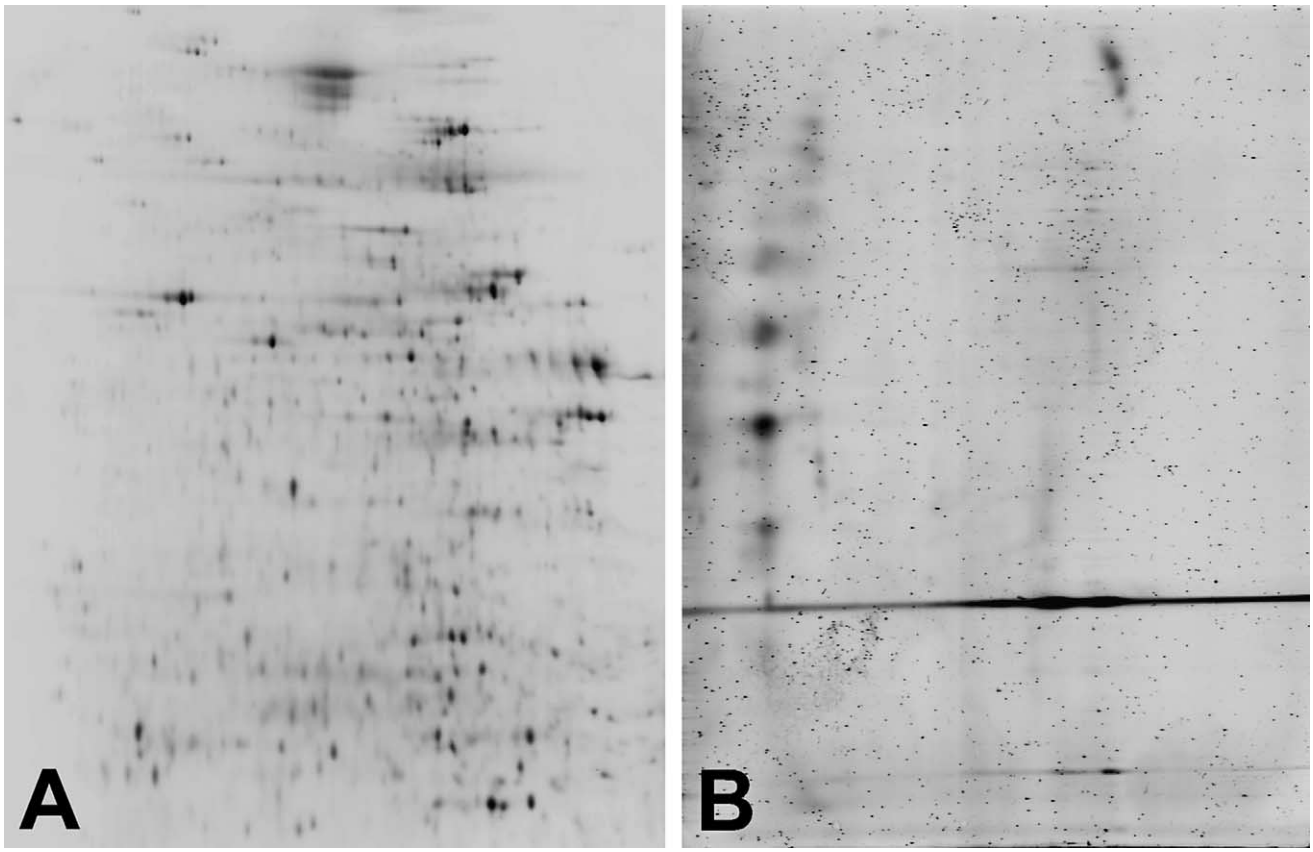

Fig. 5. 2DE of the porcine renal protein extract (A), applied to the cation-exchange sample-displacement chromatography, and of fraction 11 + 12 (B) from the sample-displacement chromatography (Fig. 4).

protein precipitation, which occurs during the calibration of the protein extract to pH 3. This experiment demonstrates that PPS can be directly applied to crude raw extracts and that the PPS results can be transferred to preparative chromatography. Furthermore parameters can be determined fastly and easily, which employ secondary interactions and therefore enlarge the possibility to find very efficient and specific chromatographic purification steps.

4. Conclusion

With PPS a large set of chromatographic parameters can be varied for the determination of optimum parameters for the chromatographic purification of biomolecules. Working in the 96-well format enables a very fast parallel processing of the PPS experiments. Therefore even the determination of parameters for non-ideal chromatography, utilizing secondary interactions is possible, which may help to establish purification steps with high specific yields. The parameters can be transferred to column chromatography including gradient and frontal chromatography. The PPS is not restricted to pre-purified samples; even crude raw extracts can be used for the parameter screening.

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