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Two separation modes combined in one column: sequential ion-exchange separation and size-exclusion chromatography of green fluorescent protein

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TWO SEPARATION MODES COMBINED IN ONE COLUMN: SEQUENTIAL ION-EXCHANGE SEPARATION AND SIZE-EXCLUSION CHROMATOGRAPHY OF GREEN FLUORESCENT PROTEIN

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ABSTRACT

Purification of green fluorescent protein from a crude solution has been investigated using a combined system of ion-exchange chromatography and size-exclusion chromatography packed into one column of a continuous annular chromatograph. Performing the chromatography with two different sorbents packed into a single column reduces transfer losses and the hold steps between the two separation steps can be eliminated. Appropriate running conditions for the continuous separation mode were achieved by searching for optimal ones in conventional batch-wise operation. Green fluorescent protein was expressed in *Saccharomyces cerevisiae*, enzymatically lysed and the extract was used as the feed stock. Superdex 200 prep grade was packed into an annular chromatograph (outer diameter: 15 cm, inner diameter: 14 cm) and on top a 1.5 cm layer of the anion-exchange sorbent Source 30 Q. Green fluorescent protein was enriched by the anion-exchange resin and further

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purified by the size-exclusion gel. Purity was analyzed by sodium dodecyl sulfonate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting, fluorescence intensity, and analytical size-exclusion chromatography.

Key Words: Continuous separation; Annular chromatography; Green fluorescent protein; Size-exclusion chromatography; Ion-exchange chromatography

INTRODUCTION

The purification of proteins from natural and recombinant sources generally requires multi-step purification protocols (1,2). An optimal purification protocol for recombinant proteins should consist of three steps, a capture, purification, and a polishing step. The capture step is designed for concentration of the product, the purification step for the removal of the main contaminants. The polishing step is for the removal of minor contaminants, aggregated, and degraded forms of the product. For capture ion-exchange and affinity techniques are very well suited. For the two other steps, all chromatographic modes are used, although ion-exchange, hydrophobic interaction, affinity chromatography, reversed phase, and size-exclusion are the preferred ones. Process chromatography is optimized regarding throughput, productivity, cycle time, purity, and yield. To increase the overall yield several strategies can be applied. Either the yield of the individual steps is improved or the number of steps is reduced. Transfer losses between two different steps can account for an overall loss of product in the range of 10% (3). Depending on the number of steps the overall yield can decrease dramatically. In case of labile proteins the speed of operations and the hold steps in between the two different steps should be minimized. Hence, the idea of combining the two separation steps into one came up. This has been proposed for conventional batch chromatography. Here we described a combination of two steps for continuous purification of proteins. The unit where this can be realized is the column of a pressurized continuous annular chromatograph (P-CAC).

The concept of a continuous chromatograph utilizing a rotating annulus was presented in the late 1940s (4). The P-CAC unit in its present form was developed at the Oak Ridge National Laboratory and improved by the company Prior Separation Technologies (Goetzis, Austria). The chromatographic apparatus consists of two concentric cylinders forming an annulus into which the resin is packed. When the system is operated as an isocratic chromatograph, the eluent is uniformly distributed at the top along the bed circumference, while the bed assembly is slowly rotated around its axis. The feed to be separated is

continuously introduced at the top of the bed at a point that remains fixed in space. The rotation of the sorbent bed coupled with the elution development induced by the downflow of eluent causes the feed components to appear as individual helical bands, each of which has a characteristic, stationary exit point (5). Synthetic protein mixtures such as hemoglobin, bovine serum albumin (BSA) as well as recombinant proteins from crude homogenates were continuously purified by annular chromatography (6–9). Metal separation with two different gels packed into one column was previously demonstrated (10). Separation of crude feed stocks containing recombinant proteins has not been described in this context. Fouling, lability of the feed, and slow mass transfer are three of several characteristics making protein separation more difficult than metal separation.

In this study we used green fluorescent protein (GFP) as a model substance, since this protein is easy to trace. Green fluorescent protein was already used as a model protein for continuous separation by annular chromatography in the previous work (8). The cDNA of GFP stems from a marine vertebrate, a Pacific Northwest jellyfish, *Aequorea victoria*. Morise et al. (1974) first identified GFP as the energy-transfer acceptor (11). Green fluorescent protein emits green light after activation of the Ca^{2+}-dependent photoprotein aequorin. So far it has been used as a reporter of gene expression, tracer of cell lineage, and as fusion tag to monitor protein localization within living cells (12). Aequorea GFP exists as monomer having a M_r of 27,000–30,000 (13). Green fluorescent protein is highly stable (14), with denaturation occurring only under very hard conditions (15). The protein can be denatured with chaotropic agents and strong acids or base ($pH < 4$ or > 11), or very high temperatures, however it will largely regain its activity when brought back to physiological conditions. In this work, we used a mutant of red shifted GFP (RsGFP) from the company Quantum (Montreal, Quebec, Canada). In comparison to the wildtype it has the gain to be brighter, that it is expressed well in a broad range of organisms and resists photobleaching (16). The cDNA of this protein was cloned into the vector pQBI63 and expressed in the yeast *Saccharomyces cerevisiae*. The clarified cell homogenate was applied as feed onto an annular chromatograph packed with two different sorbents.

MATERIAL AND METHODS

Feed Stock

The cDNA of GFP from *A. victoria*, the jelly fish, has been cloned into *S. cerevisiae*. Green fluorescent protein has been fused to the FLAG-Tag, an octapeptide with the sequence DYKDDDDK. Yeast cells were cultivated in shaking flasks using a synthetic medium (SCM^{-trp}) at the beginning and a full

medium (YPD) afterwards. At an OD between 0.6 and 0.9 cells were induced by addition of copper sulfate. Cells were harvested by centrifugation. Pellets were incubated in a sorbitol buffer supplemented with Lyticase (Sigma) for preparing yeast spheroplasts. The reduction of OD was measured at 800 nm. The complete lysis of the yeast cells was obtained by hypotone shock. The clarified supernatant was used as feed.

Preparative Anion-Exchange Chromatography in Batch Mode

The experiments were performed with an ÄKTAexplorer 100, a chromatographic system consisting of a UV-absorption monitor (UV-900), two rotarary lobe pumps (P-900), and a 25 MPa gradient mixer (M-925). Chromatographic separations are controlled by a UNICORN 3.10 control system. The anion-exchange resin Source 30 Q was packed into a HR 10 column with 10 mm diameter and 62 mm height. Workstation, controller, as well as the medium column were purchased from Amersham Pharmacia Biotech. The equilibration-, elution- and regeneration buffer were prepared from Bis-Tris supplemented with different NaCl concentration and the pH was altered to 6.5. After loading and washing with the equilibration buffer, the bound proteins were eluted with gradients of different slope.

Preparative Size-Exclusion Chromatography(SEC) in Batch Mode

Size-exclusion chromatography was performed on the same chromatography workstation as described above. Both size-exclusion resins Superdex 200 prep grade and Superdex 75 prep grade (Amersham Pharmacia Biotech) were applied for this separation. The gel was packed into a column with a diameter of 10 mm and a height of 272 mm. The column was equilibrated with a 20 m*M* Bis-Tris buffer containing 100 m*M* NaCl, pH 6.5.

Combined Ion Exchange Chromatography and Size-Exclusion Chromatography in a Continuous Annular Chromatograph

A continuous annular chromatograph from the company Prior Separation Technologies (Goetzis, Austria) was used for this study. The system was equipped with three piston pumps (VP 120) from KRONLAB (Sinsheim, Germany) for delivering eluent buffer and feed, and one peristaltic pump (P-1) from Amersham Pharmacia was used for delivering the regeneration buffer. The CAC system was composed of two concentric cylinders with an outer diameter of 15 cm and an inner

diameter of 14 cm. The size-exclusion resin Superdex 200 prep grade was filled into the annulus up to a height of 21 cm. A 1.5 cm layer of the ion-exchange medium Source 30 Q was packed on top. The Source medium was covered with glassbeads to prevent mixing of feed with eluent buffer. The eluent buffer was a 20 m*M* Bis-Tris buffer supplemented with 100 m*M* NaCl. Elution was performed with a step gradient. Ninety outletports were connected to plastic vials for sampling.

Enzyme Assay

Green fluorescent protein in the feed and the collected fractions were determined by a SPECTRAmax™ GEMINI XS Dual-Scanning Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA) measuring the fluorescence at 480 nm. A calibration curve was established with commercially available GFP from CLONTECH (Palo Alto, CA) in a range from 10 to 500 μg/mL was determined. As calibration curve a simple quadratic equation was used.

Analytical Size-Exclusion Chromatography

The purity of GFP was determined by size-exclusion chromatography (SEC) using a Superdex 75 HR 10/30 column from Amersham Pharmacia Biotech. Size-exclusion chromatography was performed on a 2150 HPLC pump (LKB, Bromma; Sweden). A 50 μL volume of each sample was applied onto the column employing an autosampler 1050 from Hewlett-Packard (Waldbronn, Germany). Constituents were eluted with 0.1 *M* sodium phosphate buffer, pH 7.0, containing 150 m*M* NaCl at a flow-rate of 1 mL/min. The UV detection at 214 nm was carried out on a 2151 variable-wavelength monitor (LKB). The UV signals were processed on a Turbochrom chromatography workstation 3.1 (PE Nelson Systems, Cupertino, CA). A calibration curve for recombinant GFP (CLONTECH, Palo Alto, CA) between 10 and 100 μg/mL was established. Different molecular mass standards (octapeptide with a M_r of 1131 Da, lysozyme, carbonic anhydrase, BSA) were used to determine the distribution coefficient (K_{AV}) of GFP.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis, Silver Stain, Western Blot

Fractions were analyzed with 4–20% acrylamide gradient gels using an XCell mini cell, both purchased from Novex Experimental Technology (San Diego, CA). Fractions were mixed with SDS sample buffer in a ratio of 1:1 and boiled for 10 min. A 10 μL volume of each sample was loaded.

Electrophoretic conditions were applied according to the manufacturer's protocol. Protein staining was performed with silvernitrate which has a 100 times higher sensitivity than Coomassie blue.

For Western blotting the proteins were blotted onto a nitrocellulose membrane using an XCell Blot module from Novex. The protein verification was obtained with the detection of the FLAG-Tag by the specific antibody anti-FLAG M1. The M1 antibody was detected using anti-mouse IgG. The real protein verification was conducted by a color reaction with BCIP and NBT.

Protein Assay

The protein was quantified by a modified Bradford assay (Bio-Rad Laboratories, Munich, Germany). The developed color was measured by a microtiter photometer at 590 nm. A calibration curve for BSA between 200 μg/mL and 2 mg/mL was determined.

RESULTS AND DISCUSSION

The goal of the study was the separation of GFP by ion-exchange chromatography and size-exclusion chromatography packed into one column. First, the operation conditions were optimized by small batch experiments. The task was to find the appropriate binding conditions for GFP. Since GFP has a pI of 5.6, an anion-exchange resin and a loading buffer with a pH value of 6.5 were chosen. Under these conditions, the GFP binds to the stationary phase and a majority of the other contaminants in the cell extract passing the column without retention. Yeast extract prepared as described under the experimental section was loaded onto the column and primarily eluted by a steep linear gradient from 0–1 *M* NaCl followed by a flat linear gradient. Fractions were collected from the flow-through, the wash, and the elution. Purity was determined by SDS–PAGE with silver staining, Western blot, and protein assay. The flow-through and the wash fraction did not contain any GFP. However, the peak pattern showed that the GFP fractions were not optimally separated from the other components. Thus, the conductivity was determined in the range before the GFP is eluted. In the next experiment the conductivity of the yeast extract and the equilibration buffer were adjusted to 11.1 mS. This buffer composition was tested in a run with a linear gradient from 100 m*M* to 1 *M* NaCl. Collected fractions were analyzed as described above. Green fluorescent protein could not be detected in the flowthrough and wash. Therefore it was decided to test a step gradient elution with the same loading conditions, since the method should be applicable in a continuous annular chromatograph.

Twenty three milliliters of extract were loaded onto the ion-exchange medium and eluted with a step gradient of 100 m*M*, 250 m*M* and 1 *M* NaCl. The buffer, containing 1 *M* sodium chloride was used for regeneration. The elution profile of the step eluent purification is shown in the chromatogram in Fig. 1. Collected fractions were analyzed by SDS–PAGE with silver staining, Western blot, protein assay and with analytical size-exclusion chromatography and fluorescence spectroscopy. The silver stain (figure not shown) and the fluorescence detection indicated an enrichment of the GFP, but the purification was not very sufficient.

Two hundred microliters of the prepurified GFP sample were loaded to the size-exclusion medium Superdex 200 prep grade. Twenty millimolar Bis-Tris buffer supplemented with 100 m*M* NaCl was the equilibration buffer, for elution a 20 m*M* Bis-Tris buffer containing 250 m*M* NaCl was used and regeneration was performed with a 20 m*M* Bis-Tris buffer supplemented with 1 *M* NaCl. In Fig. 2 a chromatogram representing the separation of GFP is shown. The purities of the eluted fractions were analyzed by SDS–PAGE with silver staining (see Fig. 3). Green fluorescent protein could be obtained in a rather pure form as seen by the electrophoresis (lanes 5 and 6). Further

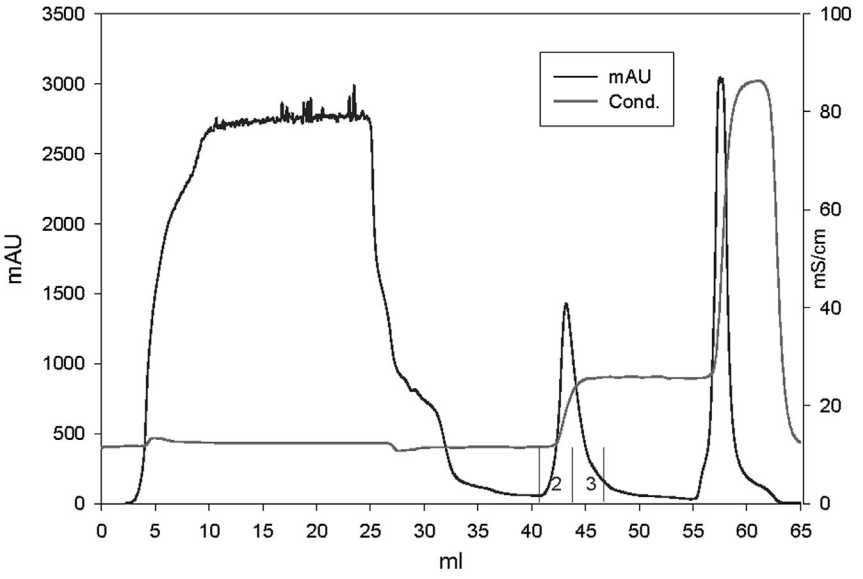

Figure 1. Step elution profile of separation of GFP from *Saccharomyces cerevisiae* lysate by anion-exchange chromatography in batch mode. Twenty three milliliters of lysate were loaded onto a 4.9 mL column. Fractions 2 and 3 contain GFP.

purity was determined by analytical size exclusion chromatography. The quantity of GFP in the collected fractions was measured by fluorescence spectroscopy. The mass balances are summarized in Table 1. The overall yield of the ion-exchange chromatography step was 92% and after size-exclusion chromatography 54%. As it can be seen from the enrichment factors and the purification factors, IEC contributes to concentrate and purify the product, while SEC only contributes to purification and not to concentration. This is explained by the nature of the separation principles. The separation principle of SEC is based on diffusion into pores and consequently the feed is diluted during separation.

The column of the annular chromatograph was packed as described above (see Fig. 4) and a schematic drawing of a 360° panorama view at the CAC column is shown in Fig. 5. The selection of the column length is mainly influenced by the available hardware. In batch a sufficient resolution could be obtained by a 20 cm SEC column. Due to the good adsorption properties of the ion-exchanger the column can be made as small as possible. The height is only determined by the handling during packing. A smaller gel could not be

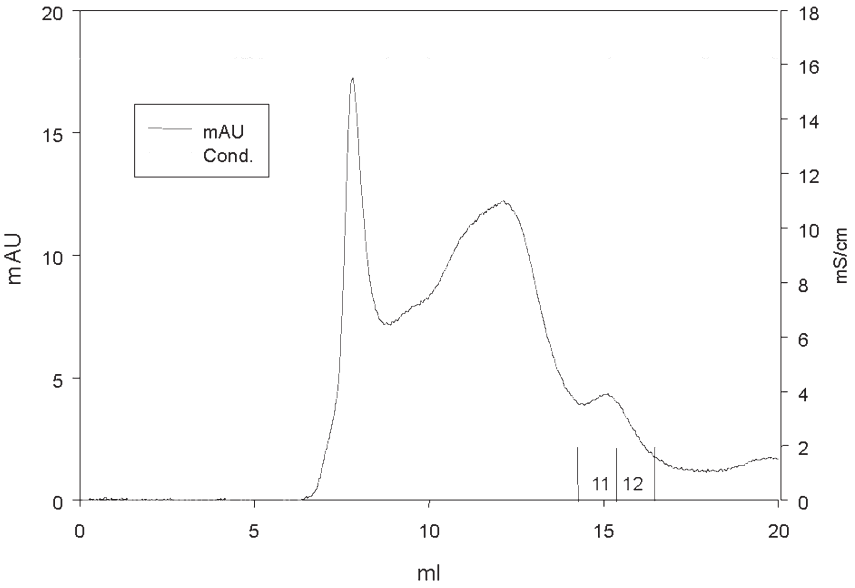

Figure 2. Chromatogram of size-exclusion chromatography using prepurified GFP as feed. A feed volume of 1% of the total column volume was introduced to Superdex 200 prep grade. Green fluorescent protein was eluted isocratically and collected in fractions 11 and 12.

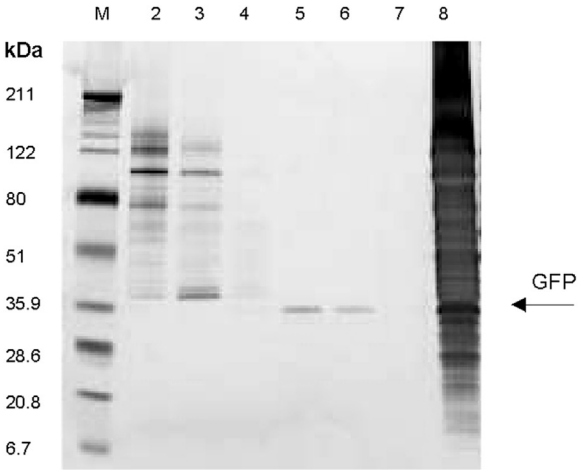

Figure 3. SDS–PAGE with silver stain of collected fractions 8–13 after size-exclusion chromatography. Lane 1: 3 μL broad range marker; lanes 2–7: 10 μL each of fraction 8–13; lane 8: 10 μL of fraction 3 from ion-exchange chromatography as comparison.

homogeneously distributed over the top of the SEC column. The system was fed with GFP and operated at four different conditions shown in Table 2. It was started with an inlet position of the salt gradient at 45°. Resolution similar to the batch experiments was not obtained. Thus shifting of the eluent inlets from 45

Figure 4. Annular chromatograph with two different separation media. At the bottom is the size-exclusion gel, above the ion-exchange resin and on top glass beads.

Table 1. Mass Balance of GFP Purification in Batch Mode. Enrichment Is Defined as the Fold Increase of Activity and Purification as the Fold Increase of Specific Activity

Step	Volume (mL)	Spec. Activity (mg GFP/mg Protein)	Activity (mg GFP/mL)	Enrichment	Purification	Yield %
Extract	23	0.06	0.37	1	1	100
IEC	6	0.22	1.23	3.36	3.67	92
SEC	0.2	0.94	0.07	0.19	15.3	54

to 135° was made to avoid contamination of the flow through with the proteins eluted with the salt step. Rotation rate of 250°/hr and flow rate of 30 mL/min of the main eluent were selected, because preliminary experiments showed that optimal resolution with these conditions (8). For a certain time, samples were collected in 90 vials and detected at 280 nm (chromatogram see Fig. 6). In all experiments, collected GFP samples were analyzed by SDS–PAGE with silver staining, Western blot, protein assay, fluorescence spectroscopy and analytical size-exclusion chromatography. The elution profile of the clarified homogenate showed three main peaks. The peak started to elute at 20° consisted of two not completely separated peaks containing yeast proteins and cell constituents. The later the elution started, the better the resolution was shown by the adjacent

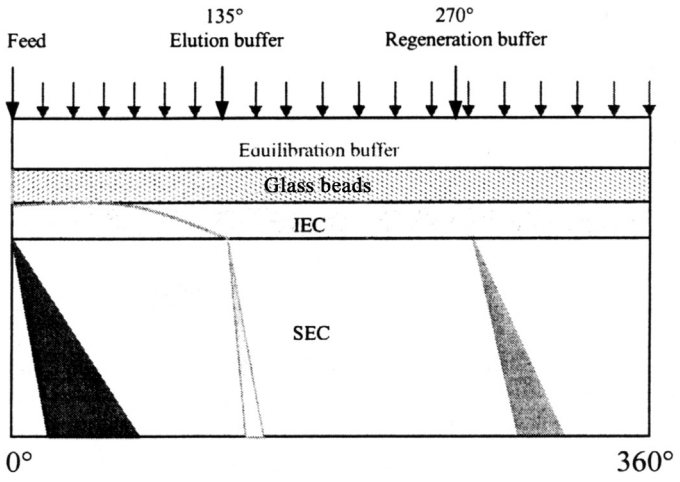

Figure 5. Schematic drawing of a 360° panorama view at an AC column.

Table 2. Operation Conditions of GFP-Separation by P-CAC Using Two Different Media Packed into a Single Column

Parameter	Run I	Run II	Run III	Run IV	Units
Feed flow rate	1	1	1	0.5	mL/min
Elution buffer flow rate	2	3	3	3	mL/min
Regeneration buffer flow rate	2	2	2	2	mL/min
Equilibration buffer flow rate	30	30	30	30	mL/min
Elution buffer inlet	45	90	135	135	degree
Regeneration buffer inlet	270	270	270	270	degree
Rotation	250	250	250	250	degree/hr

smaller peak. Thus in run III the best separation of GFP from other cell components could be obtained. The third peak that started to elute around 270° is presumably salt and small molecular mass impurities. In the first experiment the feed inlet was fixed at 0° and the inlet for the 20 m*M* Bis-Tris buffer containing 100 m*M* NaCl, the elution buffer, was fixed 45° behind the feed inlet. Green fluorescent protein was collected at 76–88°, but as the chromatogram shows in Fig. 6, the fractions where GFP could be detected did not elute in a single peak but were still integrated in the first peak. Therefore, the GFP was not efficiently separated from the major bulk. No single GFP band was shown by SDS–PAGE with silver staining (Fig. 7). In the following tests the step eluent inlet was changed from 45 to 90° and at least to 135°. Thereby a better resolution was achieved. Highest separation was obtained in the third experiment. In run III GFP was eluted at 160, 164, 168, and 172°. The mass balances of all continuous experiments are summarized in Table 3. Our experimental data (Table 3) indicate that in our case the length of the wash step influences the purity of GFP. Results of run III are subdivided into two calculations denoted as III a and III b to demonstrate a further improvement of purity by skipping the first fraction containing GFP. The only drawback is a decline in yield.

The regeneration buffer with 1 *M* NaCl was introduced to the column at 270°. Samples, collected after the regeneration step were also checked for the presents of GFP. No GFP could be detected in these fractions (data not shown), which implicates a full elution of product with 250 m*M* NaCl. Table 4 presents the angle where the GFP was eluted in the different runs and the obtained purities of GFP in each fraction.

Results of batch chromatography and continuous annular chromatography are just partly comparable, since the operation conditions have not been completely identical. Currently we do not have an understanding of the feed width of the size-

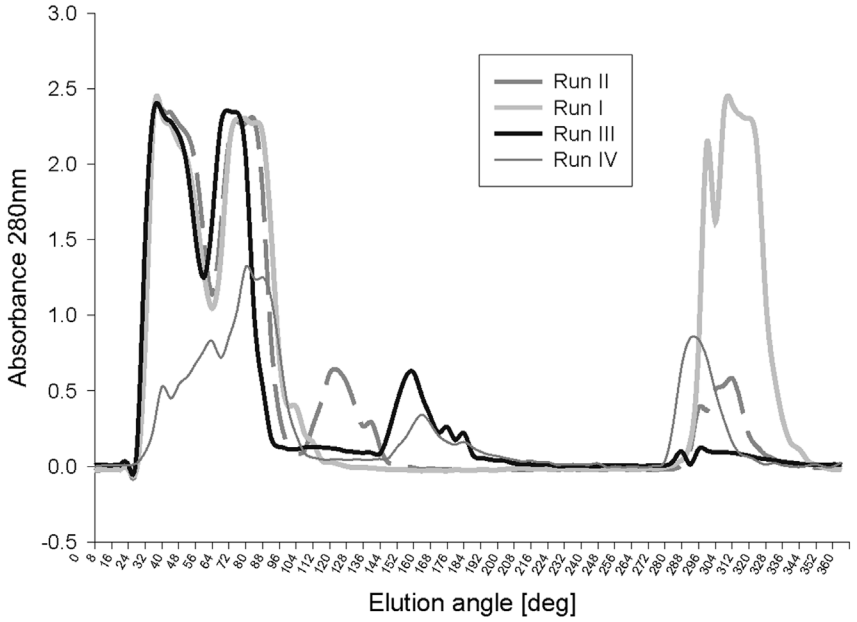

Figure 6. Chromatogram of continuous purification of GFP by annular chromatography. The purification was performed by IEC and SEC, combined in one single column. Different elution profiles are obtained at varying conditions. Best purification was achieved in run III. Green fluorescent protein was found in fractions eluted at 160–172°.

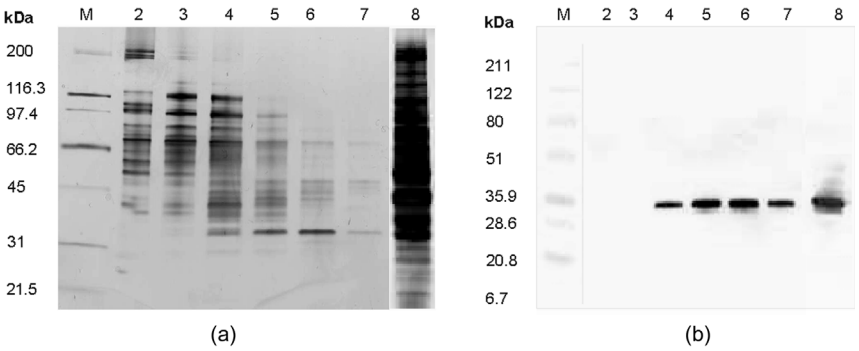

Figure 7. SDS–PAGE with silver stain (a) and Western blot using anti-Flag antibody M1 (b) of collected fractions 38–43. Lane 1 (a): 5 μL silver stain marker; lane 1 (b): 5 μL broad range marker; lanes 2–7: 10 μL of collected fractions; lane 8: 10 μL yeast extract as control.

Table 3. Mass Balance of Continuous Separation of GFP. Enrichment Is Defined as the Fold Increase of Activity and Purification as the Fold Increase of Specific Activity

Step	Spec. Activity (mg GFP/mg Protein)	Activity (mg GFP/mL)	Enrichment	Purification	Yield %
Extract	0.1	0.65	1	1	100
I	0.27	0.19	0.29	2.76	67
II	0.22	0.22	0.34	2.24	86
III a	0.24	0.38	0.58	2.45	92
III b	0.45	0.45	0.69	4.59	82
IV	0.32	0.28	0.43	3.26	100

exclusion chromatography in the continuous mode. It seems that the feed zone for SEC in the continuous mode is wider compared to the batch experiments. This is an explanation, why we lost resolution. The overall yield in the continuous mode is higher, since transfer losses and losses due to peak cutting are circumvented.

Table 4. Purity of GFP- Containing Samples Measured by Analytical Size-Exclusion Chromatography

	Elution Angle (°)	Purity GFP (%)
Run I	76	4.9
	80	28.5
	84	60.9
	88	66.2
Run II	116	6.9
	120	33.3
	124	63.4
	128	87
Run III	160	5
	164	24.5
	168	37.7
	172	91.3
Run IV	160	14.5
	164	51.1
	168	75.3
	172	85.6

Modeling of this two step process is required to ease optimization and comperative studies. The separation in the continuous mode certainly could be improved by a higher size-exclusion bed. Parameter of optimization are angular velocity, feed flow rate, eluent flow rate, and position of inlets.

ABBREVIATIONS

BCIP	5-Bromo-4-chloro-3-indolyl-phosphate-*p*-Toluidinsalt
Bis-Tris	bis (2-Hydroxyethyl) imino-tris (hydroxymethyl) methane
BSA	Bovine serum albumin
GFP	Green fluorescent protein
IEC	Ion-exchange chromatography
IgG	Immunglobulin G
K_{AV}	Available distribution coefficient
NBT	Nitrobluetetrazoliumchloride
P-CAC	Preparative continuous annular chromatograph
RsGFP	Red shifted green fluorescent protein
SCM^{-trp}	Synthetic culture medium without tryptophane
SEC	Size-exclusion chromatography
YPD	Yeast extract peptone dextrose

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