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Continuous separation of green fluorescent protein by annular chromatography

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Abstract

The concept of annular chromatography was tested by separation of a real protein solution used in biotechnology. Green fluorescent protein was expressed in *S. cerevisiae* and the extract was continuously separated by a pressurized annular chromatograph packed with a Superdex 200 prep grade size-exclusion chromatography medium. Purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blotting and fluorescent intensity. The continuous mode was compared to batchwise operation. Under the assumption that equivalent fractions are collected, both modes are comparable.

Keywords: Green-fluorescent protein

1. Introduction

The concept of continuous annular chromatography was introduced by Martin [1]. The group at Oak Ridge National Laboratory developed a system that can be operated with pressure; the pressurized annular chromatograph [2]. The company Prior Separation Technologies (Götzis, Austria) improved the design and developed a unit that meets the requirements for biotechnological production purposes. They also launched a laboratory-scale unit with an outer annular diameter of 15 cm. In principle a much smaller unit could be used as previously demonstrated [3].

Several reports were published describing the continuous separation of protein mixtures [4–6]. In all cases synthetic mixtures were investigated. So far real protein solutions were not processed by pressurized annular chromatography (P-CAC). Methods of biopharmaceutical applications must have integrated a regeneration step, the most widely applied solution for this purpose is 1 *M* NaOH. Feed streams from biotechnological origin such as culture supernatants and cell homogenates tend to accelerated fouling. Thus continuous regeneration is mandatory to prevent accumulation of contaminants and adventitious agents on the bed. After reaching saturation these compounds may be introduced into the product stream. Furthermore fouling may destroy the integrity of the packed bed and diminish the resolution.

Size-exclusion chromatography is usually avoided in the conventional batch chromatography, since the productivity is very low compared to adsorption chromatography. P-CAC offers the advantage of increased productivity in comparison to conventional modes. As model feed stream an extract from *Saccharomyces cerevisiae* transformed with an expression vector for green fluorescent protein (GFP) was used. GFP can be easily traced and quantified by fluorescence. The yeast extract can be

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considered as a worst case scenario regarding following and handling. Successful separation allows the conclusion. The P-CAC system can be applied for a big variety of biotechnological applications. Transformed yeast cells overexpressing green fluorescent protein from *Aequoria victoria*, the jellyfish [7] were mass propagated, harvested by centrifugation and enzymatically lysed. This feed stock was separated continuously by P-CAC and results were compared to conventional batch separation.

2. Material and methods

2.1. Feed stock

The cDNA of GFP from *A. victoria*, the jellyfish has been cloned into *S. cerevisiae* as described by Einhauer et al. [7]. GFP has been fused to the FLAG-tag, an octapeptide with the sequence DYKDDDDK.

Yeast cells were cultivated in shake flasks using a yeast peptone dextrose (YPD) medium. Induction was effected by addition of copper sulfate. Cells were harvested by centrifugation and enzymatically lysed according to a slightly modified method described by McDonnell et al. [8]. The clarified supernatant was used as feed.

2.2. Separation in batch mode

Superdex 200 prep grade (Amersham Pharmacia, Uppsala, Sweden) was used as separation medium. A column of 265 mm×10 mm was connected to a ProSys system (Biosepra, Marlborough, MA, USA), a flow-rate of 1 ml/min was applied, effluent was monitored at 280 nm. As eluent a 10 m*M* phosphate buffer, pH 7.0 supplemented with 150 m*M* NaCl was used.

A 500-μl volume of lysate was injected. The column was calibrated by sequential injection of standard compounds, tryptophan, cytochrome *c*, ovalbumin, bovine serum albumin (BSA), immunoglobulin G (IgG), ferritin and blue dextran.

The retention volume of blue dextran was used for calculation of the void volume.

The available distribution coefficient was calculated as:

$$K_{av} = \frac{V_R - V_0}{V_t - V_0}$$

where V_R is the retention volume of the individual substance and V_t the total column volume.

2.3. Continuous separation

Continuous separation was performed with a P-CAC system from Prior Separation Technologies.

The outer annular diameter was 15 cm, the inner annular diameter was 14 cm. Ninety outlet ports were connected to plastic vials for sampling. The column height was 22 cm. A feed inlet nozzle and an inlet port for the NaOH solution were placed at the top of the bed which was covered by glass beads. The separation was carried out with two different rotation rates at 250°/h and 500°/h.

2.4. Enzyme assay

GFP was quantified by a Lumi-Imager (Roche, Vienna, Austria). Arbitrary units (Boehringer light units, BLUs) were used for the enzyme activity. Purified recombinant GFP of a concentration of 1 mg/ml from Clontech (Palo Alto, CA, USA) was used as a reference.

2.5. Protein assay

The protein was quantified by a modified Bradford assay (Bio-Rad Labs., Munich, Germany) and BSA was used for calibration.

2.6. Electrophoretic techniques

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by a modified procedure initially described by Laemmli [9]. Precast gels from Novex (4–20% acrylamide) were used. Electrophoretic conditions were applied according to the manufacturer's protocol (Novex, San Diego, CA, USA). For Western blotting an Anti-FLAG antibody (M1, Sigma) was used. The procedure was described earlier by Schuster et al. [10].

3. Results and discussion

The separation of GFP from the crude feed stock, the clarified yeast extract was first tested in batch

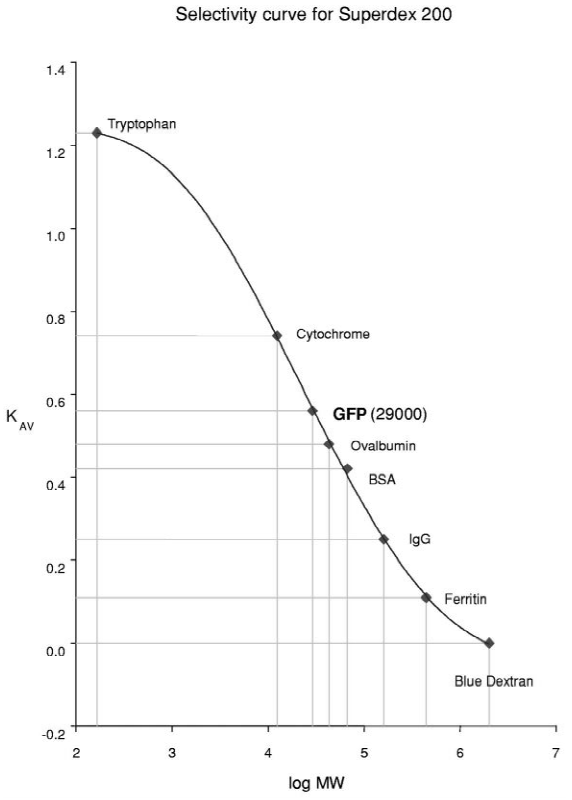


Fig. 1. Plot of the available distribution coefficient versus the logarithm of the molecular mass of standard proteins and GFP. A 500-μl volume each of blue dextran (M_r 2 000 000), ferritin (M_r 443 000), IgG (M_r 160 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), GFP (M_r 29 000), cytochrome *c* (M_r 12 400), and tryptophan (M_r 165) were loaded onto a column HR10/30 (26.5 cm×10 cm) filled with Superdex 200 prep grade. The proteins were eluted with a 10 m*M* phosphate buffer supplemented with 150 m*M* NaCl at a flow-rate of 1 ml/min.

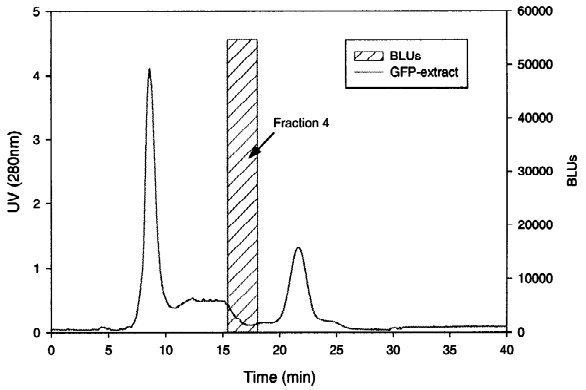
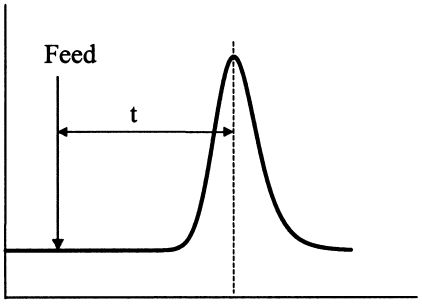


Fig. 2. Separation of GFP from *S. cerevisiae* lysate by Superdex 200 prep grade in the batch mode. A 500-μl volume of lysate was loaded onto a 20.8 ml column. The column was operated with a linear velocity of 1.27 cm/min. The hatched area indicates the pooled fractions.

chromatography experiments with Superdex 75 prep grade and Superdex 200 prep grade (data not shown). Since resolution for GFP was superior with Superdex 200 further experiments were performed with this gel. The column was calibrated by sequential injection of standard proteins. The plot of K_{av} versus molecular mass is shown in Fig. 1. GFP is located in the middle part of the plot indicating that the column will be able to retain high- and low-molecular-mass components from our molecule of interest.

In Fig. 2 a chromatogram representing the separation of GFP from crude feed stock is shown. The elution of GFP is positioned in the middle part of the UV profile.



$$\theta = \omega \cdot t$$

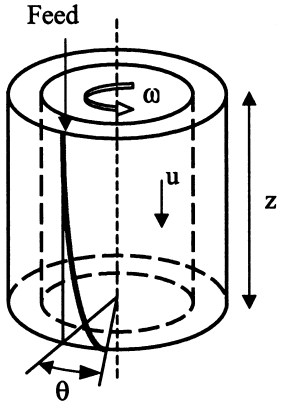


Fig. 3. Schematic drawing of the transfer of a batchwise chromatographic operation to a continuous one using annular chromatography, t=retention time, θ=angular displacement, ω=angular velocity, z=column length and u is eluent velocity.

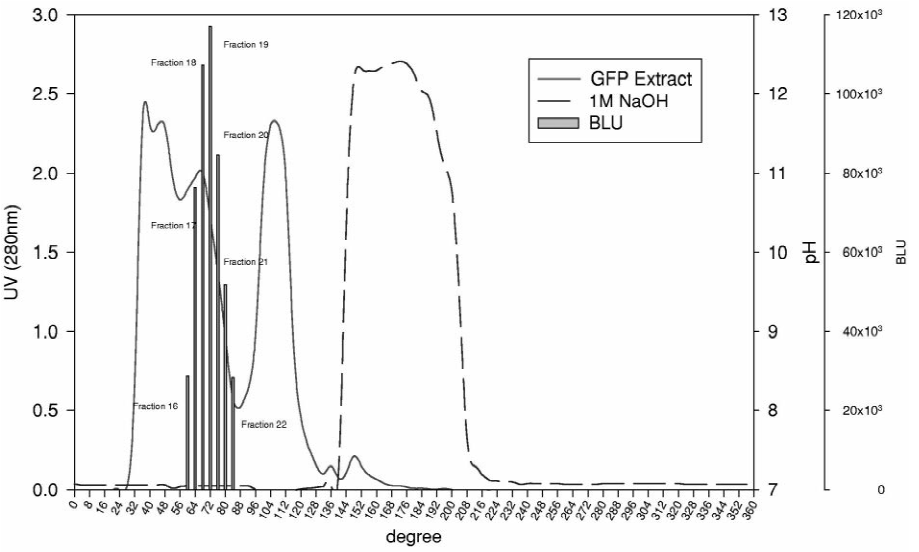


Fig. 4. Chromatogram of continuous purification of GFP from *S. cerevisiae* by annular chromatography using a rotation rate of 250°/h and an eluent velocity of 20 ml/min. The hatched area indicates the pooled fractions.

The transfer from batch mode to continuous mode is simply in case of annular chromatography. This was described by several authors [11–13]. Analogously to the retention time in batchwise operation of chromatography columns an elution angle (θ) for annular chromatography can be defined. This simple calculation (see Fig. 3) can be used for the starting point, for setting the operation parameter and is an aid to predict the elution angle.

A similar eluent velocity (1.27 cm/min) as used for the batch mode was also applied in the continuous mode (0.88 cm/min). According to Sisson et al. [12] an equivalent feed flow was used for the continuous separation. In batch mode a feed volume equivalent to 2.4% of the total column volume was applied. When transferring the operation to continuous mode the column length was kept constant. The feed flow-rate was set to 2.4% of the total flow-rate. The operation conditions are summarized in Table 1 and the chromatogram is shown in Fig. 4.

With a rotation rate of 250°/h the whole circum-

Table 1
Operation conditions for P-CAC

Parameter	Value	Units
Feed flow-rate	0.5	ml/min
Eluent flow-rate	20	ml/min
Rotation rate	250, 500	°/h
Bed length	22	cm
Bed cross sectional area	22.8	cm^2

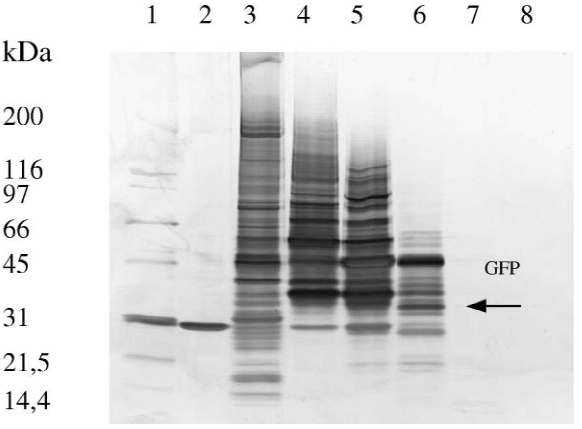


Fig. 5. SDS–PAGE with silver stain of collected fractions 1–6 from the batch separation. Lane 1: Mixture of high- and low-range marker proteins; lane 2: 15 μl pure GFP expressed in *E. coli* as positive control, and lanes 3–8: 25 μl each of fractions 1–6.

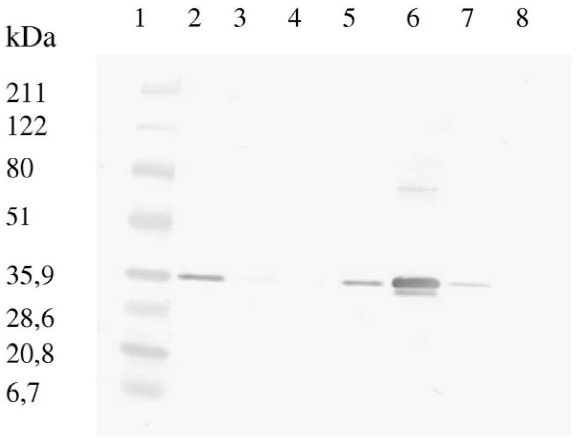


Fig. 6. Western blot using anti-Flag antibody M1 of collected fractions 1–6, lanes 3–8. Lane 1: Broad range marker; lane 2: positive control; lanes 3–8: the collected fractions. A 25-μl volume of each fraction has been loaded onto the electrophoresis gels.

ference of the annulus was not used, indicating suboptimal operation condition. Nevertheless we could show that it is possible to integrate the separation and the continuous regeneration into a single step. As already described earlier [3] the NaOH eluted over a very broad elution angle. In the NaOH pool protein could not be detected. That means that the regeneration is very efficient. Extended fouling studies were not performed. To investigate the influence of the rotation rate on the separation, the same process was performed at 500°/h keeping all other parameters constant. Fig. 9 shows the chromatogram of this run. It can be seen that this UV profile is much broader in comparison to that at 250°/h and resembles more closely to the batch chromatogram. The purity factor with 250°/h and 500°/h did not significantly differ. Theoretically a high purity is expected at a high rotation rate. Maximum resolution is already obtained at 250°/h for these particular separation conditions. At 500°/h the feed flow-rate could be further increased to increase the throughput.

The purities of the eluted fractions from batch mode were compared to the purities obtained by continuous separation using SDS–PAGE and Western blotting (see Figs. 5–8). Fractions of a lower elution angle are less pure then the fractions with higher elution angles (Fig. 9). The mass balances are summarized in Tables 2–4. In comparison the batch mode showed a lower purification factor than the continuous separation. The Western blot and silver-stain are shown in Figs. 10 and 11. This might be an artifact due to pooling of fractions. This assumption is confirmed by higher specific activity in the continuous mode.

We conclude that the transfer from batch to continuous was possible. A continuous regeneration step could be included. The separation must be carefully optimized regarding rotation rate, feed flow-rate and collection of fraction.

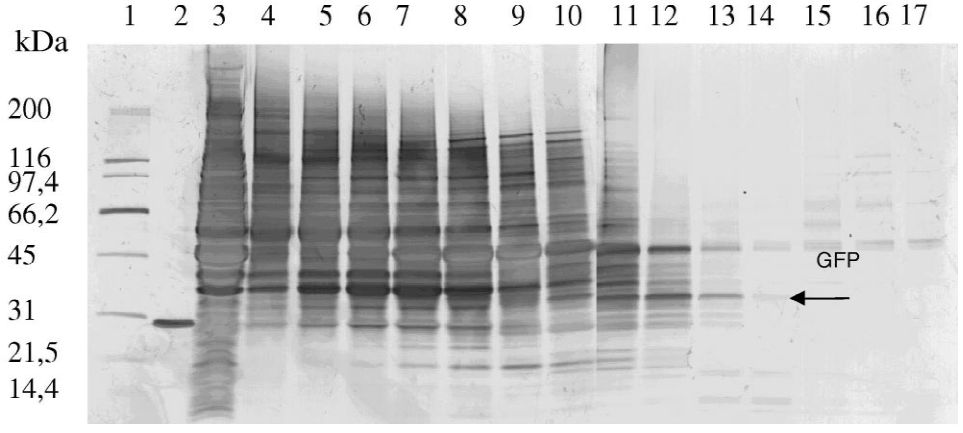


Fig. 7. SDS–PAGE with silver stain of collected fractions 14–27 from the continuous separation. Lane 1: Broad range marker; lane 2: 8 μl of the positive control (pure GFP expressed in *E. coli*); lanes 3–14: 15 μl each of fractions 14–27.

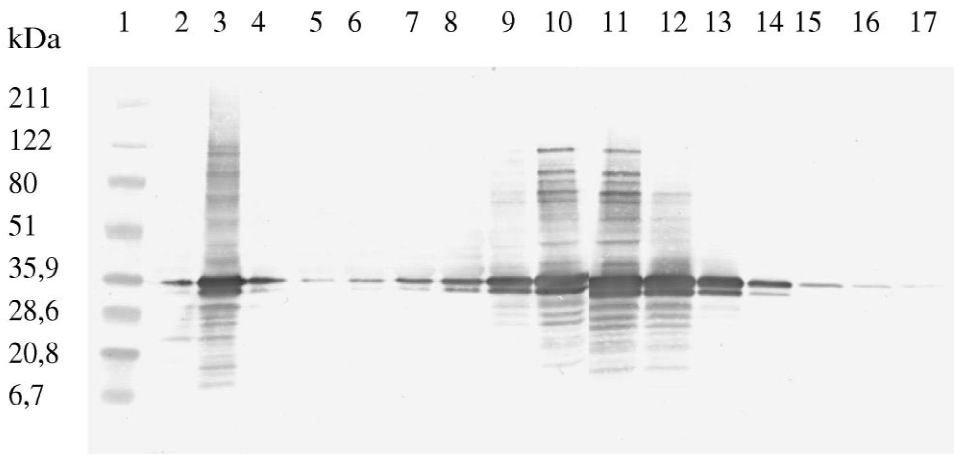


Fig. 8. Western blot using anti-Flag antibody M1 of collected fractions 14–27, lanes 4–17. Lane 1: Broad range marker; lane 2: yeast strain BJ 3505 as negative control; lane 3: GFP-extract. A 20-μl volume of negative control and 25 μl GFP-extract and fractions have been loaded onto the electrophoresis gels.

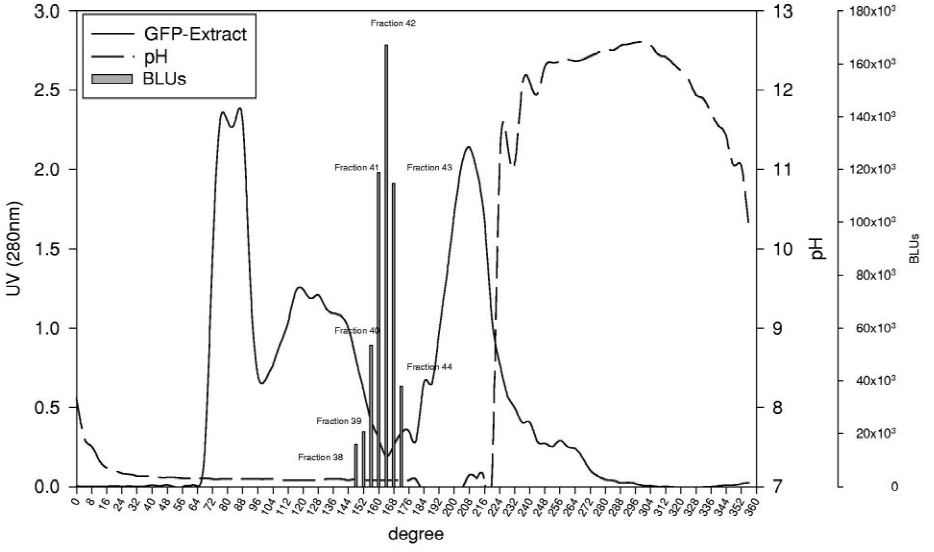


Fig. 9. Chromatogram of continuous purification of GFP from *S. cerevisiae* by annular chromatography using a rotation rate of 500°/h and an eluent velocity of 20 ml/min. The hatched area indicates the pooled fractions.

Table 2
Mass balance of batchwise operation of GFP

	Volume (ml)	Protein (mg/ml)	Protein total (mg)	GFP (μg/ml)	GFP total (μg)	Purification factor	Recovery (%)
Feed	0.5	9.4	4.7	29.6	14.8	1	100
Eluate (fraction 4)	2.5	0.1	0.25	5.6	14.5	1.85	98

Table 3
Mass balance of continuous separation of GFP at 250°/h

	Volume (ml)	Protein (mg/ml)	Protein total (mg)	GFP (μg/ml)	GFP total (μg)	Purification factor	Recovery (%)
Feed	15.7	9.4	148	29.6	466	1	100
Eluate (fractions 16–22)	49	1.6	77.9	7.3	357	2.3	77

Table 4
Mass balance of continuous separation of GFP at 500°/h

	Volume (ml)	Protein (mg/ml)	Protein total (mg)	GFP (μg/ml)	GFP total (μg)	Purification factor	Recovery (%)
Feed	15.7	9.4	148	29.6	466	1	100
Eluate (fractions 38–44)	49	1.1	55.4	7.8	381	2.5	82

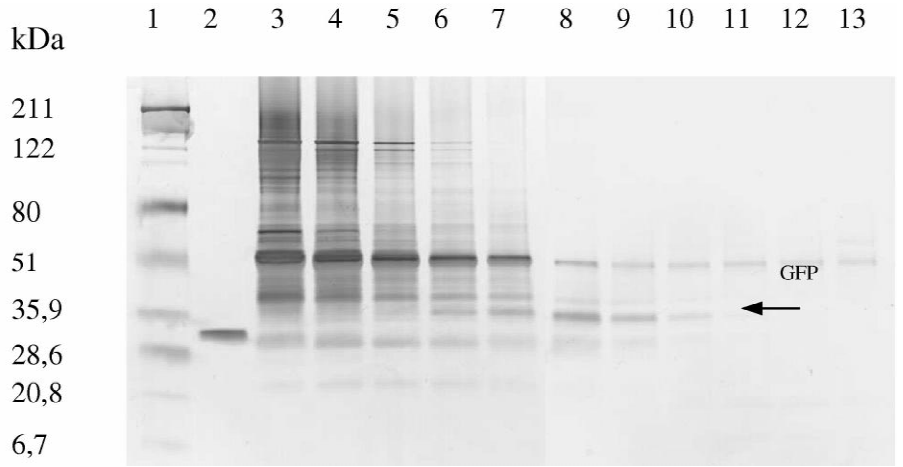


Fig. 10. SDS–PAGE with silver stain of collected fractions 36–46 from the continuous separation. Lane 1: Broad range marker; lane 2: 10 μl of pure GFP expressed in *E. coli* used as positive control; lanes 3–14: 15 μl each of fractions 36–46.

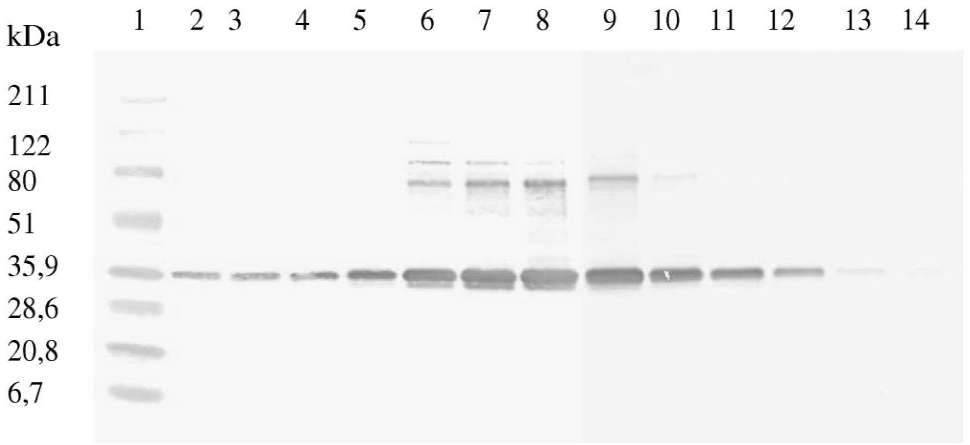


Fig. 11. Western blot using anti-Flag antibody M1 of collected fractions 35–46, lanes 3–14. Lane 1: Broad range marker; lane 2: GFP-probe as positive control. A 15-μl volume of positive control and 15 μl of each fraction have been loaded onto the electrophoresis gels.

4. Nomenclature

K_{AV}	Distribution coefficient
V_R	Retention volume, ml
V_t	Total column volume, ml
V_0	Void volume, ml
t	Retention time, min
u	Superficial velocity, cm/min
z	Column length, cm

Greek symbols

ω	Rotation rate, °/h
θ	Displacement from feed point, degree

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