Protein Engineering vol.15 no.10 pp.775–777, 2002

COMMUNICATION

Affinity chromatography system for parallel purification of recombinant protein samples

C.Gottstein[1,2] and R.Forde[3]

[1]Department of Internal Medicine I, Laboratory for Tumortherapy, University of Cologne, 50924 Cologne and [3]Aqua Research, 50974 Cologne, Germany

[2]To whom correspondence should be addressed.
E-mail: claudia.gottstein@uni-koeln.de

In protein engineering, the tasks of generating and testing a large number of variants of a molecule and of optimizing expression conditions for one distinct molecule create the need for purification methods that can handle a large number of samples simultaneously. We describe the development and some application results of a simple affinity chromatography system that can be used for the parallel purification of 24 protein samples, yielding sufficient quantities for biochemical and functional analysis. Advantages of this system over existing systems are as follows. Compared with commercially available complete chromatography systems, the costs of this system are minimal. In comparison with vacuum systems with various outlets, and with batch purification systems where centrifugation is necessary, this system allows gentler processing of the samples. This could be important for proteins that are easily damaged.

Keywords: affinity chromatography/chromatography system/expression conditions optimization/parallel protein purification/recombinant proteins

Introduction

In protein engineering, the tasks of generating and testing a large number of variants of a molecule and of optimizing expression conditions for one distinct molecule create the need for purification methods that can handle a large number of samples simultaneously. We describe the development and some application results of a simple affinity chromatography system that can be used for the parallel purification of 24 protein samples, yielding sufficient quantities for biochemical and functional analysis.

Materials and methods

Construction of the affinity chromatography system

An overview of the system is presented in Figure 1. A buffer reservoir designed to permit flow at identical flow rates to 24 circular arranged outlets is connected to 24 affinity columns. The flow is produced by gravity force and the flow rate is primarily controlled by the fluid head pressure. Adjustment of the flow rate is achieved by varying the height of the buffer fluid level above the affinity resin. The technical details are shown on the right.

The buffer reservoir was created by drilling 24 threaded holes, arranged in a circle and all positioned at the same height, in a 3 in Plexiglas cylinder; 24 chrome-plated steel nipples were screwed into the holes and sealed with Teflon tape and silicone sealant. The lower end of the cylinder was sealed off with a 4×4 in Plexiglas plate. The plate was 0.25 in thick and was attached to the cylinder with epoxy glue. Tygon tubing (Cole Parmer, London, UK) (1/16 in) was tightly fitted over the ends of the nipples and adjustable tubing clamps were positioned half way down the tubing allowing closure of the respective tubing. The clamps can be opened partially or fully, providing an additional means to control the flow rate, if desired. The lower ends of the tubings were connected to 3 ml syringes via a Luer fitting. These syringes were cut in the middle and fitted tightly on to disposable 1 ml plastic columns (Bio-Rad, Hemel Hempstead Hertfordshire, UK), thereby creating an airtight connection between the buffer reservoir and the column. The columns were filled with affinity resin, in our case Ni-NTA resin (Qiagen, Hilden, Germany). The outlets of the columns are positioned over disposable 50 ml Falcon tubes (Becton Dickinson Biosciences, Erembodegen, Belgium), which collect the flowthrough.

Purification procedure

Disposable 1 ml columns were filled with Ni-NTA affinity resin, connected to the tubings and equilibrated in running buffer (PBS, pH 7.4). Protein preparations were loaded manually on each column and the columns were reconnected with the tubings to the buffer reservoir. In some cases, a wash procedure was performed to decrease unspecifically bound protein prior to elution. To this end the respective buffers (PBS, pH 7.4, containing 0.5 M NaCl; PBS, pH 7.4, containing 10 mM imidazole) were filled into the reservoir and the columns were washed until the reservoir was almost drained. Then the clamps at the respective tubings were closed and the buffer reservoir was completely emptied and filled with the next wash buffer. This ensured that the tubings always stayed full of fluid and no air was introduced into the flow. After the last prewash step, columns were washed again with PBS (pH 7.4) and proteins were eluted manually with 50–200 mM imidazole (Sigma-Aldrich, St. Louis, MO). The first 2 ml of elution fractions contained the specific protein. The fractions were pooled and dialyzed against PBS (pH 7.4) to remove the imidazole. The affinity resins were taken out of the columns and pooled for the regeneration procedure according to the manufacturer's instructions.

Biochemical and functional testing of the purified proteins

Yields of specific proteins were measured as follows. Protein samples were subjected to scanning spectrophotometry (Figure 2A) and total protein amount was calculated from the absorption at 280 nm. Purity was assessed by SDS–PAGE and subsequent staining with Coomassie Brilliant Blue. The specific protein yield was calculated from total amount of protein and the purity of the specific protein.

The function of proteins was determined by the following assays. For the coagulation-inducing fusion protein, binding was measured by fluorescent activated cell staining (FACS) and effector function by a two-stage coagulation assay.

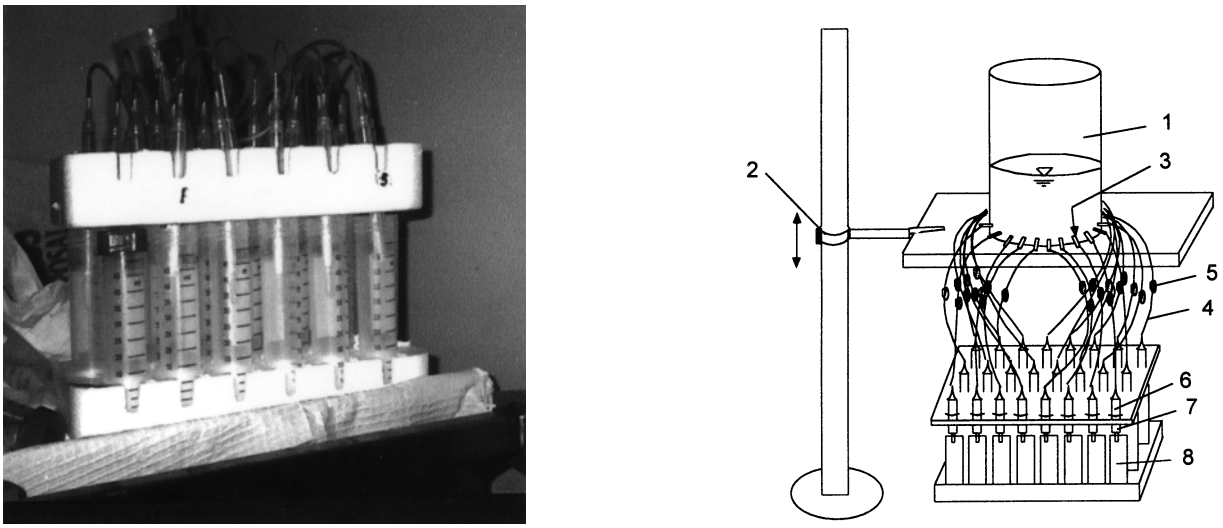

Fig. 1. Affinity chromatography system for purifying 24 recombinant proteins in parallel. The technical details are shown on the right: A buffer reservoir (1) is positioned via an adjustable stand (2) to allow gravity flow of buffer to 24 affinity columns (7). The flow path is established via 24 circular arranged nipples (3), that are connected by Tygon tubing (4) to 3 ml syringes (6), which are cut in the middle and fitted tightly on to the columns. Adjustable clamps (5) are positioned on the Tygon tubing (4) for opening or closure of the respective tubing. The columns (7) are filled with the affinity resin and the flowthrough is collected in 50 ml tubes (8).

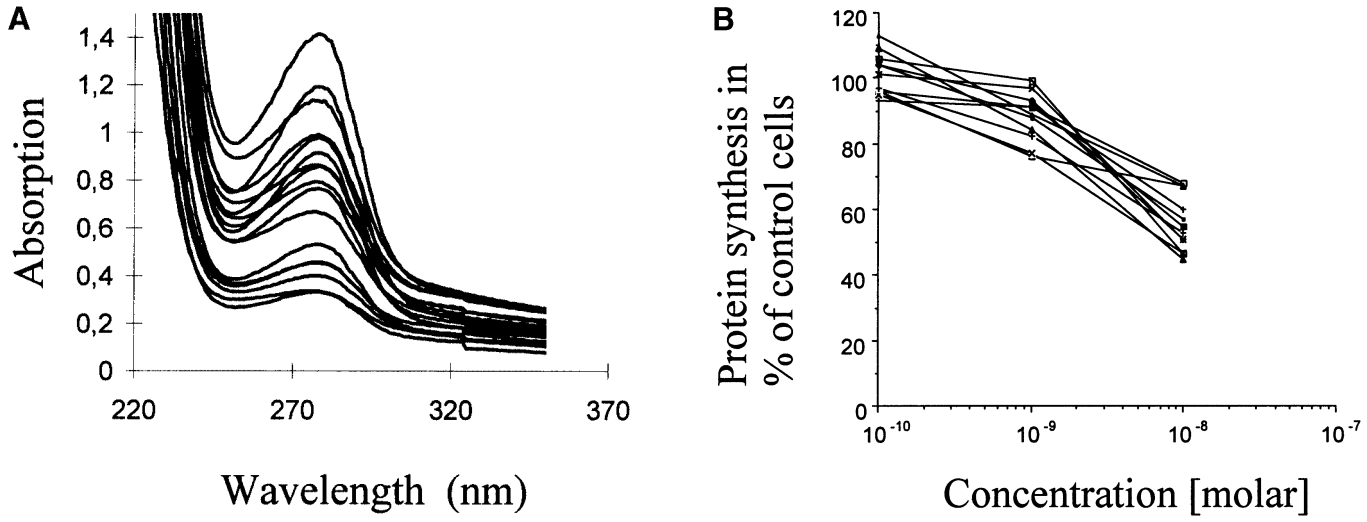

Fig. 2. Application results using the system depicted in Figure 1. (**A**) Scanning photometry to determine the concentration of 18 different fusion protein samples that were expressed under varying conditions to optimize the yield and then purified on the chromatography system shown in Figure 1. Absorption peaks are shown for the different proteins and reflect the concentration of total protein in each case. Yields of specific proteins were calculated (see text) and ranged from 4 to 80 µg. (**B**) Protein synthesis inhibition assay performed as described in the text. Samples were recombinant immunotoxins expressed under varying conditions to optimize function and then purified on the chromatography system shown in Figure 1. Target cells were incubated with purified recombinant immunotoxins at various concentrations. The protein synthesis of treated cells is given as a percentage of protein synthesis of untreated control cells. Inhibition at the highest concentration varied from 25 to 55%.

Immunotoxins were analyzed in a [^{3}H]leucine incorporation assay.

FACS

Target cells were incubated with the fusion proteins at 10 µg/ml, detected with a FITC-labeled secondary antibody and measured on a flow cytometer (Becton Dickinson Biosciences).

Coagulation assay

Coagulation function was determined as described previously (Gottstein *et al.*, 2001). Briefly, fusion proteins were incubated with a coagulation factor mix and factor X. The ability of the fusion protein to activate factor X to Xa was measured using a chromogenic substrate specific for factor Xa and measuring the increase in absorption at 405 nm.

[^{3}H]Leucine incorporation assay

This assay measures the protein synthesis of immunotoxin-treated cells in comparison with untreated control cells. It was performed as described previously (Gottstein *et al.*, 1994). Briefly, target cells were incubated with immunotoxins in triplicate at different concentrations, then ^{3}H-labeled leucine was added and after 24 h cells were harvested on a glass-fiber filter. The radioactivity of the filter pieces was measured in a

scintillation counter and the percentage of protein synthesis in comparison with protein synthesis of untreated control cells was calculated.

Application results

Application 1. Expression of a His-tagged fusion protein and optimization of yield

In this experiment, a 60 kDa fusion protein containing a His-tagged scFv fused to a coagulation-inducing protein was expressed under varying expression conditions to identify the conditions for highest yield. Nine different conditions were tested in duplicate, i.e. 18 samples were processed. Proteins were extracted via osmotic shock as described previously (Gottstein *et al.*, 2001) from 100 ml of *Escherichia coli* suspension each. The protein preparations were purified on the affinity chromatography system. Yields of specific protein ranged from 4 to 80 μg, allowing us to identify the best expression conditions regarding the yield of protein (Figure 2A). Subsequent binding and coagulation assays confirmed that the proteins were functionally active.

Application 2. Expression of His-tagged immunotoxins and optimization of function

Recombinant immunotoxins containing a 6× His-tag were prepared as described previously (Derbyshire *et al.*, 1997) and expressed under varyious conditions to identify the conditions resulting in the best functional activity. Twelve different samples were tested. Expression and purification of proteins were performed analogously to the procedure described in application 1. The concentration of the proteins was determined and a [^{3}H]leucine incorporation assay was performed. Inhibition curves are shown in Figure 2B. Inhibition of target cells at the highest concentration tested ranged from 25 to 55%.

In conclusion, we have described the construction of an affinity chromatography system that combines low cost and the possibility of purifying in a gentle process a large number of samples in parallel. This system is easy to build and allows the optimization of expression conditions of recombinant proteins. It is also suitable for purifying a number of mutation variants of a protein in order to compare their functional activities. We have used this system successfully to optimize expression conditions of various recombinant proteins.

Acknowledgements

We gratefully acknowledge the assistance of M.Unruh. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, SFB502-T6.

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Received April 5, 2002; revised July 25, 2002; accepted July 25, 2002