Single-step affinity purification of toxic and non-toxic proteins on a fluidics platform

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Received 31st August 2004, Accepted 7th December 2004
First published as an Advance Article on the web 25th January 2005
DOI: 10.1039/b413292k

Single-step fusion-based affinity purification of proteins with pH-controllable linkers was carried out in a fluidic device. The linkers were previously derived from self-splicing protein elements called inteins. Two different linkers were generated to solve two distinct separation problems: one for rapid single-step affinity purification of a wide range of proteins, and the other specifically for the purification of cytotoxic proteins. Scale-down factors of 185 resulted in separations in a 27 μl bed-volume. A rotating CD format was chosen because of its simplicity in effecting fluid movement through centrifugal force without the complications associated with electro-osmosis and other pumping methods. The design and fabrication of the fluidic device and the protein purification process are described. This work, which demonstrates the purification of active proteins by two distinct fluidic separations, is widely applicable to small-scale massively parallel proteomic separations.

Introduction

The advantages of miniaturization of bioprocessing for protein analysis from laboratory- (ml–l) to analytical- (100s μl–ml) to microfluidic-scale (μl) are numerous. The benefits include decreased sample volume, ease of automation and ability to integrate multiple processes (reaction, separation and analysis). Miniaturization also offers new opportunities, including development of devices with high surface-volume ratios for effective cooling, and the ability to use surface chemistry to advantage. Furthermore, the small length-scales have increased the importance of diffusion on reaction and mixing at very low creeping flow rates and reduced the importance of inertia. Also, strong interfacial phenomena effects allow the use of surface tension to prevent a fluid from moving, as has been exploited in a fluid valve.

The main goal of fluidics devices for bioprocessing is to control and transfer very small quantities of liquids between different unit processes on a chip, such that, after isolation and purification, the final product is of sufficient yield and purity that analysis of the quantity and activity of this product is possible. As with large-scale bioprocessing for the production of pharmaceuticals, the fewer the isolation and recovery steps the less product is lost. To this end, adsorptive processes and specifically bio-affinity capture techniques are particularly attractive since they exhibit high selectivity and can be easily miniaturized.

Various methods for moving small quantities of fluids or suspended particles in miniaturized devices are available. These include syringe and peristaltic pumps, electrochemical (with electroosmosis) methods, bubble generation and acoustic techniques, and centrifugal-driven flows. Among these fluidic propulsion methods, centrifuge-based fluidic platforms are attractive for sensing and diagnostic applications. Their use obviates the need to design sophisticated electrokinetic or mechanical pumps. Although much of the work reported in the literature on fluidics and compact discs (CD) involves mechanics (hydrodynamics and surface tension) and channel preparation), a few papers report on scaling-down well-known bio-analytical methods such as sample preparation and MALDI-MS, ELISA, PCR amplification, and fluorescence detection.

Here, we describe two different examples of fusion-based single-step protein affinity purification and recovery in a fluidics channel on a CD platform with Escherichia coli lysates. Fusion proteins have been widely used to effect efficient protein binding to pre-selected substrates. With respect to separations, they usually comprise three different domains: a binding domain (BD) that recognizes the substrate, a target protein, and a linker domain that is recognized and can be cleaved by a pre-selected protease. Linkers are usually short, less than 15 amino acids long, and are customized to be cleaved by specific proteases, such as Factor Xa or enterokinase. The main problems that have limited the use of this technology are the need to add expensive proteases to release the desired protein, cryptic cleavage of the desired protein, and the need to remove the protease after cleavage. Often the high cost of purified proteases obviates the use of this process. We and others have solved this problem by developing controllable linkers from naturally occurring autocatalytic protein elements called inteins. To demonstrate the utility and versatility of this approach, we optimized single-step affinity purification with a self-cleaving intein applied to human acidic fibroblast growth factor at the laboratory scale.

Using random and site-directed mutagenesis, we developed two different pH-controllable inteins for purification of target proteins (Fig. 1). When the pH is lowered, a “cleavage mutant” (CM) intein undergoes cleavage at the C-terminus of
the intein, to effect protein release (Fig. 1A). Similarly, a “splicing mutant” (SM) intein, allows the intein to be spliced out of the fusion context with ligation of the flanking sequences (exteins) (Fig. 1B). This latter approach is particularly useful for toxic proteins, where the SM intein, containing an affinity tag, was used to disrupt the protein, thereby rendering it non-toxic to cells in which the protein precursor is expressed. Both derivatives were tested here on a fluidics platform. The results clearly demonstrate that intein-based fusion affinity technology can be scaled down almost 200-fold, from 5 ml in the laboratory to 27 µl in a fluidics channel, to rapidly isolate and recover non-toxic and toxic proteins in active form, at high yields and with good purity.

**Experimental**

**Construction of plasmids to express fusion proteins**

Construction of the intein fusion plasmid pMAI\(^{15}\)C was described previously. This plasmid encodes a tripartite fusion of maltose-binding protein (MBP; 42 kDa), the CM mini-intein and the C-terminal DNA-binding domain of endonuclease I-TevI (130C; 13.2 kDa). pXAI\(^{15}\)C is an analogous plasmid except that MBP is replaced with a short fusion of the first 10 amino acids of MBP and the whole of the chitin-binding domain (CBD).\(^{16,17}\) Primers W1208 (5'-AAGAGGAATCTGGTAATCAGCAAAATCTCTG-GTGATC-3') and W1210 (5'-GAGGGCGATATTCGGTGAATCC-3') were used to amplify CBD by PCR with a downstream EcoRI site. Similarly, primers W1207 (5'-GGGCGGATATCTCGTGATATGG-ATACGAC-3') and W1209 (5'-GATAACCAGGAT-ATCTGGTATACCTTACCTT ACC-3') were used to generate a fragment encoding the first 10 aa of MBP from pMAL-c2X (NEB). This fragment was then fused to the CBD fragment by gene SOEing.\(^{19}\) The fused fragment (6.9 kDa) was cloned into pMAI\(^{15}\)C using EcoRI and EcoRV sites to generate pXAI\(^{15}\)C. pMAI\(^{15}\)z and pXAI\(^{15}\)z for expression of the z subunit of *E. coli* RNA polymerase (Pol z; 36.5 kDa) were generated similarly. Construction of the pTeC164::SM::CBD plasmid was described previously.\(^{17}\) This plasmid encodes endonuclease I-TevI (28.3 kDa) with the SM mini-intein inserted before residue Cys164 and CBD inserted into SM at the point where the endonuclease domain of the intein had previously been removed.\(^{20}\)

**Protein overexpression and laboratory-scale column purification**

The constructs pXAI\(^{15}\)z and pXAI\(^{15}\)C were used to evaluate the purification of standard proteins via intein cleavage, while pTeC164::SM::CBD was used for the purification of a cytotoxic protein via intein splicing. These plasmids were transformed into the *E. coli* strain BL21(DE3)pLysS for overexpression of Pol z, 130C and I-TevI, respectively. Cells containing pXAI\(^{15}\)z and pXAI\(^{15}\)C were grown to mid-log phase (OD 0.4, 600 nm) at 37 °C in L broth (2% tryptone, 1% yeast extract and 1% w/v NaCl) with 100 µg ml\(^{-1}\) ampicillin. Expression of precursor proteins Xlz and XIC was induced for 2 h by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Cells containing pTeC164::SM::CBD were grown to an OD\(_{600}\) of 0.4 at 20 °C in L broth with 50 µg ml\(^{-1}\) kanamycin, induced with 1 mM IPTG, and harvested after 2 h by centrifugation. Batch mode column purifications of these constructs were conducted as described previously.\(^{16,17}\)
Fabrication of fluidics channel

A 30 mm long, 1.5 mm wide and 0.6 mm deep fluidics channel with a barrier at one end was etched into a silicon wafer by traditional photolithography and wet etching techniques (Fig. 2). The channel (27 µl) was covered with a sheet of polydimethylsiloxane (PDMS) that contained angled reservoirs at both ends.

Purification in a fluidics channel

Chitin beads (S 6651S, 50–100 µm, New England Biolabs) were diluted 10-fold in water and loaded into the channel using a vacuum. The channels were adhered to a compact disk (Fig. 2B). A spinning device (Model WS-200-4NPP/rv, Laurell Technologies Corporation, North Wales, PA) was used to rotate the disk. Twenty bed-volumes of pH 8.5 buffer (500 mM NaCl, 20 mM AMPD, 20 mM PIPES, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100) was passed through the channel at 250 rpm to equilibrate the chitin resin. Fifty milligrams of bacterial cells were resuspended in 1 ml chitin column buffer (500 mM NaCl, 20 mM AMPD, 20 mM PIPES, 2 mM EDTA, 1% Triton X-100 and 1 mM DTT at pH 8.5) and lysed by sonication. After centrifugation in a microcentrifuge at 14,000 rpm for 5 min, the clarified cell lysate was loaded with intermittent spinning into the feed reservoir of the channel, and the flow-through fluid was collected from the product reservoir into a microfuge tube until ten bed-volumes of lysate had passed through the channel. The beads were then washed with twenty bed-volumes of pH 8.5 buffer. Three bed-volumes of either pH 6.0 (cleavage) or pH 7.5 (splicing) buffer were applied to shift the pH from 8.5. A rotational speed of 180 rpm was used for the cell lysate loading process and 250 rpm was adopted for the washing and elution steps. The fluidics channel was then sealed with parafilm. After 24 h, 100 µl of pH 6.0 or 7.5 buffer was used to elute the protein from the beads in the channel. Eighty microliters of the eluate was concentrated to 15 µl (Savant 210A Speed-Vac, GMI, Inc., Albertville, MN) and resolved by SDS-PAGE. The remainder of the sample (20 µl) was used for the activity assay. The entire procedure was conducted at 4 °C.

Protein concentration

Protein concentration was determined after electrophoresis of purified proteins using a fluorescent stain (SYPRO® Ruby Protein Gel Stain, Cambrex–Biotherapeutic Products, East Rutherford, New Jersey). The gel was scanned with a fluorescent imager (FluoroImager, Molecular Dynamics, Sunnyvale, CA), and a software package (Imagequant 5.1, Molecular Dynamics) was used to calculate the quantities of the protein in the eluted samples. Serial dilution of target proteins of known concentration was used for calibration.

Cleavage activity assay

A DNA cleavage assay was used to test the activity of I-TevI as previously described. Briefly, ScaI-linearized plasmid DNA containing the I-TevI recognition sequence (250 ng) was incubated with samples containing purified I-TevI at 37 °C for 15 min. Samples were electrophoresed through 1% (w/v) agarose gels in 40 mM Tris-acetate (pH 8), 2 mM EDTA and DNA was visualized after staining with ethidium bromide.

Binding activity assay of 130C

Band-shift experiments were conducted to test the DNA-binding activity of 130C as previously described. Briefly, the protein was incubated for 15 min at 23 °C with 32P-end-labeled target DNA in 50 mM Tris-HCl (pH 8.0), 20 µg ml⁻¹ poly
in the fluidics channel, a mass balance calculation for recovery of precursor (XIC) using affinity binding and controlled cleavage was characterized and it has an apparent binding constant ($K_a$) of 9.6 nM. Therefore a DNA-binding assay was performed to test the activity of the 130C recovered in the eluate stream from the fluids channel purification. A native gel that demonstrates a mobility shift upon binding of 130C to its DNA substrate is shown in Fig. 4B. Comparison of the DNA-binding activity of 130C from the fluids purification with that of 130C purified by traditional chromatography shows them to have equivalent activity, verifying that proteins purified in this way retain biochemical activity.

**Intein-mediated fluidic channel purification of cytotoxic I-TevI by insertional inactivation and protein splicing**

The model cytotoxic protein is I-TevI, an intron-encoded DNA endonuclease, which kills the cells presumably because it cleaves the DNA of *E. coli* non-specifically. In this case I-TevI was inactivated by insertion of SM, the controllably splicing mini-intein. Affinity purification was achieved via CBD which was itself inserted into the SM intein. The pTevC164::Δ1-SM::CBD derivative was used to test purification by intein-mediated protein splicing on chitin beads in the fluids channel (Fig. 1B). Purification steps 2 and 3 are essentially the same as those used for the intein cleavage process except that a splicing buffer at pH 7.5 is used instead of the cleavage buffer at pH 6.0. Fractions from a fluids purification of I-TevI were resolved by SDS-PAGE and are shown in Fig. 5A. As with the laboratory-scale column experiments (lanes 1–4), three protein species are clearly visible for the fluids-scale experiments (lane 7). They are full-length ligated I-TevI (28.3 kDa), generated by splicing, and the N-terminal catalytic domain (18.9 kDa) and the C-terminal DNA binding domain (9.4 kDa) of I-TevI generated by aberrant cleavage. For quantitation, the micro-fluidic eluate can be compared with material purified in a column. From such comparisons, the concentration of I-TevI in the eluate averages approximately 0.07 mg ml$^{-1}$ (data not shown). This is equivalent to approximately 0.7 mg l$^{-1}$ culture
which compares well with the yield from the column preparations, which was approximately 0.2 mg l⁻¹ culture.¹⁷

To verify the endonuclease activity of the I-TevI endonuclease, a DNA cleavage assay was performed (Fig. 5B). Comparison of the endonuclease activity of I-TevI purified using the same technology on a column or a fluidics channel shows they have a similar specific activity. These experiments demonstrated not only that this cytotoxic protein can be purified in small scale, but also that it is highly active.

Conclusions

Intein cleavage and splicing technology have been successfully downsized 185-fold from the laboratory scale to a fluidics channel on a rotating compact disk. Success was demonstrated using two model proteins for cleavage (the α subunit of RNA polymerase of *E. coli* and the C-terminal DNA-binding domain of intron endonuclease I-TevI). Additionally, the model cytotoxic protein, endonuclease I-TevI, was successfully purified in active form from a fluidics channel using intein splicing. Further downsizing by several orders of magnitude, by further miniaturizing the channels and using more sensitive detection methods such as silver staining and Western blotting, is feasible.

Integrated fluidic systems have been developed to automate protein digestion, peptide separation and protein identification.²² They are promising to serve as the front end of mass spectrometry for automated protein analysis.²³ This fluidic separation system can potentially be integrated with other microfabricated devices to develop on-chip enzymatic assays.²⁴ The compact disk-like microfluidic platform has been used for ion analysis,²⁵ cell lysis²⁶ and multiple parallel enzyme-linked immunosorbent assays have been shown to be feasible on this platform.²⁶ With the recent commercialization of the CD technology,²⁷ the fluids protein purification system presented in this paper can be easily integrated to perform parallel processing for protein purification and identification.
This study serves as a successful example for combining intein technology with CD technology.

Acknowledgements

This work was supported by the National Institutes of Health (GM44844) and the National Science Foundation (CTS-0304055), and a Howard P. Isermann fellowship through the Department of Chemical Engineering, Rensselaer Polytechnic Institute to J.M. The authors acknowledge the contributions of the Wadsworth Center Molecular Genetics Core facility and thank John Dansereau for conducting the band-shift assay of 130C and other technical support. We also thank Carol Lyn Engel and Matt Stanger for helping with laboratory-scale column purification of I-TevI and gel quantification, respectively, and Steven Hanes for assisting with the optical micrograph (Fig. 2B).

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