Published in final edited form as:

Anal Biochem. 2009 October 15; 393(2): 176–181. doi:10.1016/j.ab.2009.07.009.

Fully automated protein purification

DeMarco V. Camper and Ronald E. Viola*

Department of Chemistry, University of Toledo, Toledo, OH 43606, USA

Abstract

Obtaining highly purified proteins is essential to begin investigating their functional and structural properties. The steps that are typically involved in purifying proteins can include an initial capture, intermediate purification, and a final polishing step. Completing these steps can take several days and require frequent attention to ensure success. Our goal was to design automated protocols that will allow the purification of proteins with minimal operator intervention. Separate methods have been produced and tested that automate the sample loading, column washing, sample elution and peak collection steps for ion-exchange, metal affinity, hydrophobic interaction and gel filtration chromatography. These individual methods are designed to be coupled and run sequentially in any order to achieve a flexible and fully automated protein purification protocol.

Keywords

protein purification; aspartate semialdehyde dehydrogenase; ÄKTA Explorer; automated purification protocols

The isolation and purification of a protein of interest is an essential first step before detailed functional and structural characterization studies can commence. The first protein purification protocols were devised in an era when cloning and over-expression of target proteins was not routinely available. Under these conditions production of purified proteins required laborious, multi-step protocols [1], that in many cases yielded less than 20% of the initial levels of the protein of interest that was present in the cells or tissues [2–6]. The development of routine techniques for protein over-expression altered the field of protein purification by providing enriched sources of proteins of interest at levels in the crude cell extracts that approaches or exceeded the levels achieved after earlier purifications. Subsequent purifications required much fewer steps thereby producing highly purified proteins in much higher yields [7].

To further improve protein purification efficiencies affinity chromatography approaches were developed in which metal chelates [8,9] or specific ligands [10–12] were immobilized on activated resins and used to selectively bind and separate the target proteins. Subsequent incorporation of specific tags [13] onto the proteins of interest changed affinity chromatography from an exotic technique to a routine purification protocol [14,15]. Now, columns designed to specifically recognize these attached tags have become the method of choice, and initially held the promise of one-step purification from crude cell extract to homogeneous protein. In reality achieving this promise is a rare event. Non-specific binding

© 2009 Elsevier Inc. All rights reserved.

*Corresponding author. Phone: 419 530 1582, Fax: 419 530 1583, E-mail address: ron.viola@utoledo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of proteins with surface exposed histidines from the cellular milieu frequently results in several (or more) proteins co-eluting from these affinity columns along with the protein of interest [16]. In addition, metal ion binding proteins have been shown to bind to IMAC resins with high affinity [17]. Methods to eliminate non-specific binding [18–20] and to separate the other proteins that bind to these affinity resins [21] have resulted in some improvements, but still have not completely eliminated these problems. As a consequence, the purification of proteins of interest frequently requires one or more chromatography steps coupled to affinity chromatography in order to achieve a highly purified protein [22,23]. In addition, in some cases the incorporation of affinity tags can have a detrimental impact on protein structure and function and must therefore be avoided.

Several automated protocols have recently been developed to streamline the protein purification process. Each of these protocols are designed around an affinity chromatographic purification, either in a one-step, high-throughput approach [24] or with affinity chromatography as the first step in pre-set dual-column protocols [25,26]. However, all of these applications are designed to work exclusively with tagged proteins, and they also lack the flexibility of allowing the user to select the type and the order of chromatographic steps. We have designed a modular approach to the purification of both tagged and untagged proteins that allows the user to select both the type and the sequence of chromatographic protocols and also to adjust the peak selection criteria that is used in each protocol.

Materials and methods

Instrumentation

The development of automated protein purification protocols was conducted by using an ÄKTA Explorer 100 FPLC (GE Healthcare) enclosed in a 4.0 °C chromatography chamber. The ÄKTA Explorer is equipped with multiple automated features, including the capability of collecting peaks with various commands, buffer and pH scouting, and shaped elution gradients. The addition of an IV-980 valve provides the capability of running additional sequential elution gradients. Enzymatic assays were carried out by using a Cary 50 UV-visible spectrophotometer (Varian). SDS-PAGE was used to confirm the molecular weight and purity of the proteins of interest.

Cell transformation and protein expression

An *Escherichia coli* cell line, BL21(DE3), was used for transformation and protein expression. Standard transformation protocols were used with this cell line (Novagen) to incorporate plasmids containing the *asd* gene from *Vibrio cholerae* (pET41a) and from *Streptococcus pneumoniae* (pET28a). Colonies obtained from these transformations were used to inoculate overnight cultures, and these cultures were then used to inoculate 1L of LB media containing the appropriate antibiotic. After growth in a shaker at 37 °C for 2–3 h, or until an OD₆₀₀ between 0.6–0.8 is reached, target protein expression was induced with 1 mM IPTG and cells were grown for an additional 4 h at 33 °C. Cells were centrifuged at 10,000 rpm at 4 °C for 2 minutes and stored at –80 °C until ready for use.

Method queue

Method Queue is a program in the ÄKTA software suite that allows multiple separation protocols to be linked together and run in sequence to automate multi-step processing between protocols. The Method Queue has significant flexibility in the linking of protocols, either running sequential protocols automatically or allowing additional criteria to be evaluated after each protocol before proceeding to the next command.

Enzyme assay

The activity of the test proteins, aspartate β -semialdehyde dehydrogenases (ASADH) from different bacterial species, was followed in the non-physiological direction by measuring the production of NADPH at 340 nm that occurs as aspartate semialdehyde (ASA) is oxidatively phosphorylated to β -aspartyl phosphate (β -P-asp) (Scheme 1).

A typical ASADH assay [27] was conducted in 120 mM Ches buffer, pH 8.5, in the presence of 0.25 mM NADP, 40 mM potassium phosphate and 200 mM KCl. After addition of the enzyme the reaction was initiated by the addition of 0.4 mM ASA.

Results

Test proteins for automated purification

Two proteins that had previously been purified through optimized manual methods were chosen to develop and test fully automated protocols for protein purification. ASA dehydrogenase from *V. cholerae* was overexpressed in *E. coli* and then purified in nearly 70% overall yield despite requiring three separate chromatography steps [7]. Ion-exchange chromatography was used as the initial capture step, followed by intermediate purification using hydrophobic interaction chromatography (HIC) and then a final gel filtration polishing step to produce highly purified enzyme that was subsequently used for crystallization and structural characterization studies [28]. The *asd* gene from *S. pneumonia* that encodes for an ASA dehydrogenase in this organism was also cloned into *E. coli* in a vector containing a carboxyl-terminal hexa-histidine tag to facilitate purification. Chromatography on a cobalt-immobilized metal affinity column (IMAC) was optimized through the use of a wash buffer containing low levels of imidazole, followed by elution with an imidazole gradient. However, a subsequent ion-exchange chromatography step was required to produce the highly purified protein that led to the first structure of an ASA dehydrogenase from a gram-positive microorganism [29]. Each of these manual methods resulted in highly purified proteins, but required frequent operator attention over a period of 2–3 days to complete each purification.

Automated protein purification criteria

Devising a flexible and automated approach for the multi-step purification of proteins provides many obvious advantages. Achieving this goal requires developing protocols with the following features for any single chromatography step:

1. the automatic triggering of elution gradients after unbound proteins are washed from the column.
2. automatic sensing and collecting of protein peaks.
3. termination of the chromatography run after the target protein has been collected.

To couple these individual protocols into a fully automated multi-step purification protocol requires several additional features:

1. the capacity to automatically load the collected protein peak onto a subsequent column
2. the ability to run unique column washing, protein elution and peak collection procedures for subsequent chromatography runs.
3. the capacity to change the protein sample conditions (buffer, pH, salt content, etc.) between chromatography steps.
4. the means to couple consecutive chromatographic protocols together to run in an automated, unattended fashion.

These criteria and features have been used to develop a fully automated approach to protein purification, and this approach has been applied to the purification of these two test proteins, one tagged and one untagged, that each required different combinations of chromatographic steps to achieve highly purified samples.

Automated purification of ASA dehydrogenase from *Vibrio cholerae*

The multi-step purification of *V. cholerae* ASA dehydrogenase (*vcASADH*) was carried out by using this fully automated approach, without any operator intervention beyond triggering the system to load the crude cell extract and then collecting the purified protein at the end of the run. After cell growth and protein expression as described in Methods the cells were resuspended in Buffer A (25 mM potassium phosphate, pH 7.0, with 1 mM EDTA and 1 mM DTT) and sonicated in an ice water bath for 5 minutes. The protein was centrifuged for 25 minutes at 11,000 rpm and 4 °C and the supernatant filtered. The protocol was initiated to load the filtered crude cell extract onto a Q Sepharose XL ion-exchange column with an air sensor detecting when sample loading was completed. This triggered implementation of the first chromatographic step, an ion-exchange protocol. This protocol washes the column with a maximum of four bed volumes of buffer A, but will automatically truncate column washing when the absorbance (A_{280}) achieves a stable baseline value. A linear salt gradient then commences from 0 to 100% buffer B (buffer A plus 1.0 M KCl) to elute the bound proteins. The protein peak of interest was automatically collected through an outlet valve (F3) with the assistance of an absorbance watch command that switches the column flow to peak collection once the absorbance at 280 nm increases above a set value (Fig. 1A). Peak collection is terminated and the flow directed back to waste when the absorbance decreases below a pre-set threshold value.

The next step in the purification of *vcASADH* involves a hydrophobic interaction chromatography (HIC) column. Because proteins typically have a higher affinity for a hydrophobic resin in the presence of high levels of kosmotropic salts, a buffer exchange step was needed to prepare the protein collected from ion-exchange chromatography for the hydrophobic column. A gel filtration column was used to accomplish this buffer exchange. Once the gradient elution was terminated the protein peak collected from the ion-exchange column was automatically loaded onto a Sephadex G-25 size exclusion column that had been pre-equilibrated with buffer C (50 mM Hepes, pH 6.0, with 1 mM EDTA, 1 mM DTT, and 1.2 M ammonium sulfate). To avoid the use of a large bed volume column that would lead to significant dilution of the protein the sample was loaded onto a small gel filtration column in multiple aliquots. This is straightforward to accomplish in the automated procedure by stacking multiple buffer exchange protocols. For each aliquot addition to the column the conductivity watch command was used to trigger diversion of the flow from the collection vessel (F4) to waste once the conductivity of the eluant drops below a preset value (in this case 75 mS/cm) which indicates that the lower salt buffer is starting to elute (Fig. 1B).

After the buffer exchange was completed, the pooled and collected protein peak was automatically loaded onto a phenyl-Sepharose hydrophobic interaction column. Detection of complete loading by an air sensor triggered the next step, an HIC protocol. This protocol washes the column with buffer C until the absorbance decreased below a threshold value and then automatically begins a decreasing linear elution gradient from 0–100% buffer D (buffer C minus the ammonium sulfate) until the protein peak is eluted (Fig. 1C). Once again the eluting protein was detected by an increase in A_{280} and, in this case, the column flow was diverted to a fraction collector to allow the selection of individual fractions to be pooled, concentrated and stored.

The results from the fully automated multi-step purification of *vcASADH* are summarized in Table 1. This automated protein purification protocol produced nearly 60 mg of highly purified

enzyme in greater than 70% overall yield by coupling ion-exchange chromatography, buffer exchange and hydrophobic interaction chromatography steps over a total period of about 8 h without requiring any operator intervention. The purified protein was concentrated to about 20 mg/mL, exchanged into the crystallization buffer conditions and stored at -20°C . Subsequent crystallization screening of this protein sample resulted in diffraction quality crystals that are comparable to those obtained from the manually purified protein.

Automated purification of ASA dehydrogenase from *Streptococcus pneumonia*

The manual purification of polyhistidine-tagged *S. pneumonia* ASA dehydrogenase (*spASADH*) required an additional chromatography step after cobalt-IMAC purification to achieve highly purified enzyme that could be crystallized [29]. This multi-step purification protocol has also been fully automated. After growth and protein expression cells were resuspended and sonicated in buffer A (50 mM Hepes, pH 7.0, with 300 mM NaCl) in an ice water bath to extract the soluble proteins. The extract was centrifuged for 25 minutes at 11,000 rpm and 4°C and then filtered with a $0.8\text{ }\mu\text{m}$ syringe filter. The filtered cellular extract was loaded onto a Talon® cobalt affinity column followed by the first protocol, an IMAC purification protocol, after automatic sample loading. This protocol washes the column with buffer A containing 10 mM imidazole until the absorbance (A_{280}) decreases below a threshold value and is stable, followed by elution with a linear gradient of buffer B (buffer A plus 200 mM imidazole). The imidazole competes with the poly-histidine tag on the protein thereby allowing the protein to be eluted from the column, but the high levels of imidazole in buffer B leads to considerable background absorbance that tends to mask the protein absorbance. This required using a concentration watch command in the IMAC protocol to trigger collection of the protein peak in the region where the peak was known to elute (from 20% to 70% of buffer B), with the peak collected through an outlet valve (F4) (Fig. 2A).

At this stage in the manual purification the protein solution was dialyzed overnight to remove the high levels of imidazole before running the ion-exchange chromatography step. Failure to carry out this buffer exchange precluded protein binding to the anion exchange resin. To replace the overnight dialysis a buffer exchange/desalting protocol was inserted into the protocol by using a size exclusion column. The protein peak collected from the cobalt-IMAC column was automatically loaded from the collection vessel onto a Sephadex G-25 column pre-equilibrated with Buffer C (20 mM Hepes, pH 7.0, with 50 mM NaCl, 0.5 mM EDTA and 1 mM DTT). Once again, to avoid the use of a large bed volume desalting column that would lead to significant dilution, the protein was loaded in small aliquots (9–10 ml) onto a 25 ml bed volume column to achieve desalting. This typically required about 8–10 repetitive runs depending on the sample volume, but again this procedure was fully automated by stacking multiple gel filtration protocols. Since the purpose of this step is to desalt the protein prior to running ion-exchange chromatography, the watch command for peak collection at this stage is based on an increase in conductivity to trigger the end of the protein peak and the beginning of the salt peak (Fig. 2B). The protein peak was collected through the appropriate outlet valve (F4). Once the conductivity went above 10 mS/cm this was an indication that the salt had begun to elute from the column, so the column flow was switched back to waste. After flushing the column with buffer C until the conductivity decreased to a baseline value this protocol was repeated for the necessary number of cycles to completely load and desalt the protein. The protein peak collected from each desalting cycle was accumulated in the same vessel.

The final polishing stage of this purification utilized a high resolution Source 30Q anion exchange column. The protein peaks pooled from the desalting column were automatically loaded onto a 25 mL Source 30Q anion exchange column by using air sensing that signals complete sample loading and triggered an ion-exchange protocol. This protocol washes the column with a maximum of four bed volumes of buffer D (50 mM Hepes, pH 7.0, with 1 mM

EDTA and 1 mM DTT). The purified protein was eluted from the column with a linear salt gradient from 0% to 100 % buffer E (buffer D plus 650 mM NaCl) that was automatically triggered after the absorbance decreased below a threshold value which indicated that all of the unbound proteins had been removed from the column (Fig. 2C).

The results from this fully automated, multi-step purification of *sp*ASADH are summarized in Table 2. A total of 21 mg of highly purified enzyme was obtained in greater than 40% overall yield without requiring any operator intervention through successive affinity chromatography, gel filtration and ion-exchange chromatography steps that were completed in about 12 h. The highly purified *sp*ASADH protein was concentrated to 16–20 mg/mL and stored at –20 °C. Once again the protein purified by this automated protocol yielded diffraction quality crystals that are indicative of the high purity achieved.

Samples of the protein fractions from each step in these automated protocols for the purification of *vc*ASADH and *sp*ASADH were visualized by Coomassie staining on an SDS-PAGE (Fig. 3) and show the increasing quality of these protein samples that was achieved at each stage in the purification.

Discussion

The need to obtain highly purified samples to carry out detailed functional and structural studies of proteins has led to many improvements in the selectivity and efficiency of purification protocols. Automated protocols have been developed that can allow rapid, high-throughput affinity purification of up to 60 samples per day by using a single chromatographic step [24]. However, obtaining the highest level of purification frequently requires multi-step protocols that will decrease the overall sample throughput. Several automated multi-step protocols have been developed that each utilize an initial affinity-tagged purification step, followed by several generic pre-set protocols that can be selected to produce milligram quantities of multiple proteins per day with >90% purity [26,30].

Our goal was to develop a fully automated, multi-step purification process that would be both time and cost efficient, and will utilize an approach that can be customized to purify any protein of interest from crude cell extracts to homogeneity without requiring any operator intervention. In contrast to the previously published protocols that were designed to maximize sample throughput, our aim was to compress multi-step purifications into a fully automated overnight run to produce highly purified protein without sacrificing either overall yield or the final sample quality. A typical multi-step protein purification usually takes several days or more and requires frequent operator intervention to switch to fraction collection during peak elution, to identify and pool the active fractions and to then prepare the pooled sample to be manually loaded onto the next column. The protocol that we have developed allows the program to select, isolate and collect the peaks of interest from each chromatographic run and automatically load them onto the next column.

Optimized automated protein purification

The automated purification of ASA dehydrogenase from *V. cholerae* (*vc*ASADH) summarized in Table 1 uses the same ion-exchange and hydrophobic interaction chromatographic steps that were used in the previously optimized manual purification. The overall protein yield, the purity of the final product, and the quality of the protein crystals achieved were the same as had been obtained from the manual purification. Besides the obvious advantage of requiring no operator intervention to complete the purification, several other features of this automated protocol aided in streamlining the purification process. Once the unbound proteins were washed from the ion-exchange column and a stable baseline was achieved the salt gradient was immediately initiated (Fig. 1A) rather than continuing to wash the column with a defined volume of buffer.

Also, in the manual purification protocol the bound proteins were eluted from the ion-exchange resin by running a 0 to 1.0 M KCl gradient, however the protein of interest was found to elute during the early part of the gradient. In the automated protocol once the protein peak was collected the gradient was terminated at less than 35% of buffer B (Fig. 1A) and the next step in the protocol was initiated.

For the histidine-tagged ASA dehydrogenase from *S. pneumonia* (spASADH) affinity (IMAC) chromatography produced purified but still not completely pure protein (Table 2). A buffer exchange was required to remove the high levels of imidazole before the final ion-exchange chromatography step, and this was accomplished by gel filtration. Again, the salt gradient was automatically initiated to elute the bound proteins from the ion-exchange column once a stable baseline was achieved during column washing. This gradient was terminated at about 30% of buffer B once the purified protein peak had been collected (Fig. 2C).

The flexibility of our purification approach allows the direct conversion of previously optimized manual purification protocols to a fully automated protocol without requiring alterations in the type or sequence of chromatographic steps or any significant modifications in how each chromatography column is run. Individual chromatographic methods have been developed to run ion-exchange, hydrophobic interaction, immobilized metal affinity and gel filtration chromatography, along with several buffer exchange methods. These methods can easily be adapted to run any type of chromatography, and the selection criteria for triggering elution gradients and peak picking can be tailored to the particular requirements of any protein purification problem. These chromatographic methods and coupling protocols have been developed by using software written for a specific line of FPLC systems and can be used directly on any instrument running this software. However, the approach used to couple optimized methods, along with methods developed to automatically change sample conditions between chromatography runs, are of general utility and can be readily adapted to other chromatography systems.

The success in automatically purifying new proteins to homogeneity starting from crude samples depends to some extent on the properties of the target protein and on the nature of the contaminating proteins. For target proteins that contain chromophoric components monitoring the eluted samples at those wavelengths will easily identify the appropriate peak for collection even in a complex protein mixture. If the target proteins are expressed at levels that make it the most abundant single protein component then the threshold values in the methods can be adjusted to recognize and collect the peak containing the desired protein. If there are multiple peaks with sufficient absorbance that elute from a column then each of those peaks can be automatically collected in different vessels. Continuing the automated purification protocol will then require prior knowledge about which eluted peak contains the protein of interest so that the next protocol can be set to load the appropriate sample onto the subsequent column. New purification protocols can be rapidly developed by selecting the appropriate methods and then coupling them together.

Conclusions

Separate protocols have been developed to carry out ion-exchange, affinity, hydrophobic interaction and size exclusion chromatography. With the inclusion of protocols for desalting and for buffer exchange it is now possible to link the chromatographic purification steps in any order to achieve fully automated and optimized purification of a target protein.

Abbreviations used

ASA, aspartate semialdehyde; ASADH, aspartate β -semialdehyde dehydrogenase; HIC, hydrophobic interaction chromatography; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl- β -thiogalactoside; *sp*ASADH, *Streptococcus pneumonia* ASADH; *vc*ASADH, *Vibrio cholerae* ASADH.

Acknowledgments

The authors thank the technical support staff at GE Healthcare for assistance in accessing and utilizing the full capabilities of the AKTÄ chromatography system.

References

1. Karsten WE, Hunsley JR, Viola RE. Purification of Aspartase and Aspartokinase-Homoserine Dehydrogenase I from *Escherichia coli* by Dye-Ligand Chromatography. *Anal. Biochem* 1985;147:336–341. [PubMed: 3893216]
2. Glaser L, Brown DH. Purification & properties of D-glucose-6-phosphate Dehydrogenase. *J. Biol. Chem* 1955;216:67–79. [PubMed: 13252007]
3. Olive C, Levy HR. The Preparation and Some Properties of Crystalline Glucose 6-Phosphate Dehydrogenase from *Leuconostoc mesenteroides*. *Biochemistry* 1967;6:730–736. [PubMed: 4381644]
4. Givot IL, Smith TA, Abeles RH. Studies on the Mechanism of Action and the Structure of the Electrophilic Center of Histidine Ammonia Lyase. *J. Biol. Chem* 1969;244:6341–6353. [PubMed: 5354960]
5. Englard S, Kaysen G, Avigad G. 5-Keto-D-fructose. VI. A Specific Reduced Nicotinamide Adenine Dinucleotide Phosphate-Linked Reductase from Yeast. *J. Biol. Chem* 1970;245:1311–1318. [PubMed: 4392628]
6. Kohn LD, Packman PM, Allen RH, Jakoby WB. Tartaric Acid Metabolism. V. Crystalline Tartrate Dehydrogenase. *J. Biol. Chem* 1968;243:2479–2485. [PubMed: 4297261]
7. Moore RA, Bocik WE, Viola RE. Expression and Purification of Aspartate β -semialdehyde Dehydrogenase from Infectious Microorganisms. *Protein Expr. Purif* 2002;25:189–194. [PubMed: 12071715]
8. Porath J, Carlsson J, Olsson J, Belfrage G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 1975;258:598–599. [PubMed: 1678]
9. Sulkowski E. Purification of proteins by IMAC. *Trends Biotechnol* 1985;3:1–7.
10. Cuatrecasas P. Protein Purification by Affinity Chromatography. *J. Biol. Chem* 1970;245:3059–3065. [PubMed: 5432796]
11. Ostrove S. Affinity chromatography: general methods. *Methods Enzymol* 1990;182:357–371. [PubMed: 2314248]
12. Ostrove S, Weiss S. Affinity chromatography: specialized techniques. *Methods Enzymol* 1990;182:371–379. [PubMed: 1690329]
13. Pryor KD, Leiting B. High-Level Expression of Soluble Protein in *Escherichia coli* Using a His₆-Tag and Maltose-Binding-Protein Double-Affinity Fusion System. *Protein Expr. Purif* 1997;10:309–319. [PubMed: 9268677]
14. Schmitt J, Hess H, Stunnenberg HG. Affinity purification of histidine-tagged proteins. *Molec. Biol. Reports* 1993;18:223–230.
15. Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S. Comparison of affinity tags for protein purification. *Protein Expr. Purif* 2005;41:98–105. [PubMed: 15802226]
16. Bolanos-Garcia VM, Davies OR. Structural analysis and classification of native proteins from *E. coli* commonly co-purified by immobilised metal affinity chromatography. *Biochimica et Biophysica Acta* 2006;1760:1304–1313. [PubMed: 16814929]

17. Kung CC, Huang WN, Huang YC, Yeh KC. Proteomic survey of copper-binding proteins in *Arabidopsis* roots by immobilized metal affinity chromatography and mass spectrometry. *Proteomics* 2009;6:2746–2758. [PubMed: 16526091]
18. Janknecht R, de Martynoff G, Lou J, Hipskind RA, Nordheim A, Stunnenberg HG. Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proc. Natl. Acad. Sci. USA* 1991;88:8972–8976. [PubMed: 1924358]
19. Westra DF, Welling GW, Koedijk DGAM, Scheffer AJ, The TH, Welling-Wester S. Immobilised metal-ion affinity chromatography purification of histidine-tagged recombinant proteins: a wash step with a low concentration of EDTA. *J. Chromatogr. B* 2001;760:129–136.
20. Franken KL, Hiemstra HS, Van Meijgaarden KE, Subronto Y, Hartigh JDen, Ottenhoff TH, Drijfhout JW. Purification of His-tagged proteins by immobilized chelate affinity chromatography: The benefits from the use of organic solvent. *Protein Expr. Purif* 2000;18:95–99. [PubMed: 10648174]
21. Jiang W, Hearn MT. Protein Interaction with Immobilized Metal Ion Affinity Ligands under High Ionic Strength Conditions. *Anal. Biochem* 1996;242:45–54. [PubMed: 8923963]
22. McCluskey AJ, Poon GMK, Garipey J. A rapid and universal tandem-purification strategy for recombinant proteins. *Protein Sci* 2007;16:2726–2732. [PubMed: 17965191]
23. Coulombe P, Meloche S. Dual-tag prokaryotic vectors for enhanced expression of full-length recombinant proteins. *Anal. Biochem* 2002;310:219–222. [PubMed: 12423642]
24. Steen J, Uhlen M, Hober S, Ottosson J. High-throughput protein purification using an automated set-up for high-yield affinity chromatography. *Protein Expr. Purif* 2006;46:173–178. [PubMed: 16483795]
25. Bhikhabhai R, Sjoberg A, Hedkvist L, Galin M, Liljedahl P, Frigard T, Pettersson N, Nilsson M, Sigrell-Simon JA, Markeland-Johansson C. Production of milligram quantities of affinity tagged-proteins using automated multistep chromatographic purification. *J. Chromatogr* 2005;1080:83–92.
26. Sigrell JA, Eklund P, Galin M, Hedkvist L, Liljedahl P, Markeland-Johansson C, Pless T, Tortenson K. Automated multi-dimensional purification of tagged proteins. *J. Struct. Funct. Genomics* 2003;4:109–114. [PubMed: 14649294]
27. Karsten WE, Viola RE. Chemical and kinetic mechanism of aspartate- β -semialdehyde dehydrogenase from *Escherichia coli*. *Biochim. Biophys. Acta* 1991;1077:209–219. [PubMed: 1673060]
28. Blanco J, Moore RA, Kalabeswaran V, Viola RE. A structural basis for the mechanism of aspartate- β -semialdehyde dehydrogenase from *Vibrio cholerae*. *Protein Sci* 2003;12:27–33. [PubMed: 12493825]
29. Faehnle CR, Liu X, Coq JLe, Viola RE. An Examination of Key Intermediates in the Catalytic Cycle of Aspartate Semialdehyde Dehydrogenase from a Gram-positive Bacteria. *J. Biol. Chem* 2006;281:31031–31040. [PubMed: 16895909]
30. Kenig M, Peternel S, Gaberc-Porekar V, Menart V. Influence of the protein oligomericity on final yield after affinity tag removal in purification of recombinant proteins. *J. Chromatogr* 2006;1101:293–306.

Fig. 1.

Automated purification of ASA dehydrogenase from *Vibrio cholerae*. (A) Q Sepharose XL ion-exchange chromatogram using absorbance monitoring and peak collection through outlet value F3. (B) Sephadex G-25 buffer exchange chromatogram using conductivity monitoring and peak collection through outlet valve F4. (C) phenyl-Sepharose hydrophobic interaction chromatogram using conductivity monitoring with the protein peak collected in fractions. Protein elution was monitored by absorbance (in milliabsorbance units) at 280 nm (red), with the salt gradient (green) or the conductivity profile (blue) overlaid on the chromatograms.

Fig. 2.

Automated purification of ASA dehydrogenase from *Streptococcus pneumonia*. (A) Cobalt-IMAC purification chromatogram using absorbance monitoring and peak collection through outlet valve F3. (B) Sephadex G-25 purification chromatogram with conductivity monitoring and peak collection through outlet valve F4. (C) Source 30Q ion-exchange chromatogram with absorption monitoring and the protein peak collected in fractions. Absorbance, salt gradient and conductivity are as indicated in Fig. 1.

Fig. 3. SDS-PAGE of automated protein purification. (A) *V. cholerae* ASA dehydrogenase purification: Lane 1, MW markers; Lane 2, crude *vcASADH*; Lane 3, Q Sepharose XL peak; Lane 4, pooled phenyl-Sepharose fractions. (B) *S. pneumoniae* ASA dehydrogenase purification: Lane 1, MW markers; Lane 2, crude *spASADH*; Lane 3, cobalt-IMAC peak; Lane 4, pooled Source 30Q fractions.

Scheme 1.
Activity Assay of ASA Dehydrogenase

Table 1
Automated purification of ASA dehydrogenase from *Vibrio cholerae*

protocol	Total Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Extraction	3590	152	23.6	100	1.0
Ion-exchange (Q Sepharose XL)	3370	95	35.6	94	1.5
Hydrophobic (Phenyl Sepharose)	2640	58	45.3	73.4	1.9

Table 2
Automated purification of ASA dehydrogenase from *Streptococcus pneumoniae*

protocol	Total Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Extraction	1340	71.0	18.9	100	1.0
Affinity (Cobalt-IMAC)	1210	42.9	28.3	90.5	1.5
Ion-exchange (Source 30Q)	569	21.0	46.4	42.4	2.5