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Identifying Competitive Protein Antagonists for F-Actin with Reverse-Phase HPLC

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Abstract

F-actin binding constants are traditionally determined by centrifugal cosedimentation with actin microfilaments, where bound protein is separated from actin with SDS-PAGE and quantitated using densitometry. Here, we demonstrate that UV quantitation of reverse-phase HPLC separated proteins provides increased accuracy and sensitivity, can be fully automated, and allows one to perform F-actin competition assays on similar sized proteins.

Keywords

Actin; Villin; Dematin; Headpiece; HPLC; Protein

Main Text

As is true of most, if not all, actin-based structures, the *in vivo* order and function of the macromolecular complex cannot be attributed to the *in vitro* assembly properties of actin, but instead are dependent on its associated actin binding proteins [1]. Therefore, it is of great scientific interest to determine both the F-actin binding site and affinities for the greater than 60 distinct families of F-actin binding proteins [2]. F-actin binding constants are most commonly determined with an F-actin sedimentation assay (Supp. Fig. 1; Reviewed in [3]). Traditionally, the amount of ligand present in the pelleted fraction (protein bound to F-actin) is quantitated densitometrically by comparing the staining intensity of its respective band to a standard series run on the same gel. Although, this is a viable technique for determining binding constants of certain proteins, the SDS-PAGE based methodology is burdened by several limitations discussed below. In addition to electrophoresis, F-actin binding constants have also been determined by measuring the loss of radioactivity (of a radio-labeled ligand) from the supernatant [4], changes in fluorescence of chemically modified actin, and even ligand specific assays, like ATPase activity in the case of myosin (Reviewed in [3]).

The use of HPLC to quantitate F-actin sedimentation assays is not an entirely novel concept and has been employed by others to measure F-actin binding constants for proteins that are either too small to be analyzed with gel-electrophoresis [5] or have the same effective molecular

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COMPETING INTERESTS STATEMENTS

The authors declare that they have no competing financial interests.

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weight as actin [6], as well as to determine the protein stoichiometry of macromolecular complexes bound to actin [7]. In this report, we demonstrate that, in addition to both increasing accuracy and sensitivity as well as permitting full automation, an HPLC-based methodology provides a means of demonstrating that two, similarly sized proteins share the same F-actin binding site.

SDS-PAGE separated proteins were quantitated in a similar fashion to that described by Meng *et al.*, 2005 [8]. Briefly, a standard series of villin headpiece in SDS-PAGE loading buffer (10 mM Tris, pH 7.5, 2.5% glycerol, 0.5% Sodium Dodecyl Sulfate, 1% Beta-Mercaptoethanol) was run through an SDS-PAGE tricine gel [9]. Following electrophoresis, each gel was fixed for 20 minutes with 0.5% glutaraldehyde, acid precipitated with 12% trichloroacetate for 1 hour, and then stained with a colloidal preparation of either R-250 or G-250 coomassie brilliant blue [10]. The gels were then scanned and quantitated using ImageJ software [11]. The HPLC response curve was obtained by injecting a standard series of villin headpiece in 7% acetic acid (with a Waters 717Plus Autosampler) onto an analytical C18 reverse phase column (Vydac 218TP3410) and eluted with a linear gradient of acetonitrile (20 to 80% in 60 minutes) at a flow rate of 0.75 mL/min (Waters 600E Pump/Controller). Trifluoroacetate (0.1%) was used as the ion-pairing agent. The area under each eluting peak, measured by absorption at 222 nm (recorded on a Shimadzu SPD-10A), was quantitated using Waters Empower[®] software. For unknown reasons, appreciable amounts of actin, but, importantly, not villin-type headpiece domains, was found stick to the column and elute on a subsequent blank runs (Supp. Fig. 2) and therefore actin was not quantitated. Both the SDS-PAGE and UV/HPLC methodology utilized a similar volume of protein per run (10-15 μ L).

Figure 1, displays both the raw data and resulting response curves for a standard series quantitated with either SDS-PAGE or HPLC. It may be appreciated from Figure 1C that the SDS-PAGE based methodology is not linear with increasing protein concentration. The non-linearity is exacerbated at higher concentrations, where the shallower slope makes this methodology intrinsically less accurate. Importantly this effect is not due to saturation of the scanner (Fig. 1B). In contrast, UV-quantitation of eluting HPLC peaks produces a highly linear response (Fig. 1D) that extends over several orders of magnitude (Supp. Fig. 3). The UV/HPLC data is also cleaner as there is no vertical offset (background staining), gel artifacts (i.e. solvent front), or user bias (position and extent of the band; Fig. 1A) and this methodology circumvents the use of surface active chemicals like SDS, for which pipetting is intrinsically inaccurate. Furthermore, the fact that coomassie staining intensity varies significantly between gels, requires that a standard series be run on each gel, limiting the number of samples that can be run and prohibits the averaging of multiple, independent measurements. The lack of both artifacts and any practical limitation on the number of samples and standards that can be run in the HPLC-based allows simple arithmetic averaging between runs. We routinely obtain R^2 -values in excess of 0.995 from standard series of a variety of different villin-type headpiece domains.

Although, an increase in accuracy and the ability to fully automate the process is beneficial, the true strength of our reverse-phase HPLC-based methodology lies in its ability to resolve proteins based on subtle differences in physicochemical characteristics instead of size. Although it has long been assumed that the several homologous villin-type headpiece domains, most notably villin (VHP) and dematin headpiece (DHP), share the same F-actin binding site [12], the nearly identical size of these two constructs (VHP: 67 residues, 7.6 kDa; DHP: 68 residues, 7.9 kDa) has prevented us and others from testing this hypothesis. Here, we show that with reverse-phase HPLC, it is possible to individually resolve and therefore simultaneously quantitate VHP and DHP, which allowed us to coincubate these two proteins with F-actin in order to test whether these two proteins compete with one another for the same F-actin binding site.

Equation 1 is an equilibrium binding expression describing two proteins (X , Y ; termed competitive antagonists) that compete with one another for the same binding site (A). From Eq. 1, it is possible to derive Eq. 2 (Supplemental Material, [13, 14]), where X is the protein of interest, Y is a competitive antagonist, B_{max} is the number of binding sites, and NS is non-specific binding, from which one can compute the theoretical amount of each protein bound as a function of its concentration and the concentration of a competitive antagonist. As should be expected, when the dissociation and non-specific binding constants are refined to the data in Fig. 2B,C (via a numerical least squares regression), we obtain similar values to those previously determined for each protein in isolation.

$$[X_{bound}] = \frac{B_{max} \frac{[X_{total} - X_{bound}]}{K_{d,X}}}{1 + \frac{[X_{total} - X_{bound}]}{K_{d,X}} + \frac{[Y_{total}]}{K_{d,Y}}} + NS_X \cdot [X_{total} - X_{bound}] \quad (2)$$

In summary, we have shown that reverse-phase HPLC offers a significant increase in both accuracy and sensitivity, can be fully automated, permits arithmetic averaging of sequential runs, and most importantly allows one to perform F-actin competition assays between similarly sized proteins. This later point will likely be very relevant as researchers are taking advantage of the modular nature of most F-actin binding proteins and therefore are likely to encounter situations where they are dealing with two, similarly sized constructs. Furthermore, since the binding sites of most F-actin binding proteins are clustered into to a few “hot-spots” about the surface of actin [15], by way of an F-actin competition assay (performed between a protein, whose F-actin binding site has been determined, and one, whose binding sites is unknown), a rough idea of the binding site may be obtained without the use of electron microscopic reconstructions. Finally, there is no technical reason, why this methodology would not be applicable to other equilibrium cosedimentation assays, including those involving either myosin or microtubules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Comparison of the raw data and standard curves produced with SDS-PAGE to those quantitated with our UV/HPLC-based methodology. **A)** Representative SDS-PAGE tricine gel of a standard series of villin headpiece. **B)** Three dimensional plot of pixel intensity of **(A)** as a function of x,y translation. The resulting signal plotted as a function of protein concentration for SDS-PAGE, stained with colloidal suspension of either coomassie R-250 (triangles) or G-250 (circles), are displayed in **C** and those quantitated with UV/HPLC are displayed in **D**. Inset: Stack plot of UV chromatographs from a standard series of villin headpiece.

Figure 2.

F-actin competition assays preformed between villin and dematin headpiece. F-actin (20 μM) was incubated with a constant concentration of DHP (10, 35, or 100 μM) while the concentration of villin headpiece was increased from 0 to 200 μM . **A)** Representative chromatographs from an F-actin competition assay preformed between DHP (35 μM) and increasing concentrations of VHP (0, 5, 10, 25, 50, 100, and 200 μM). The amount of either villin or dematin headpiece bound to F-actin is plotted against the total concentration of villin headpiece in **B** and **C**, respectively. Solid lines are refined theoretical curves for pure competitive antagonism (Calculated from Eq. 2; $K_{d,VHP} = 10.9$ μM ; $NS_{VHP} = 1.9\%$; $K_{d,DHP} = 6.4$ μM ; $NS_{DHP} = 5.1\%$).