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Technical note

Separating full-length protein from aggregating proteolytic products using filter flow-through purification

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ABSTRACT

Separation of full-length protein from proteolytic products is challenging, since the properties used to isolate the protein can also be present in proteolytic products. Many separation techniques risk non-specific protein adhesion and/or require a lot of time, enabling continued proteolysis and aggregation after lysis. We demonstrate that proteolytic products aggregate for two different proteins. As a result, full-length protein can be rapidly separated from these fragments by filter flow-through purification, resulting in a substantial protein purity enhancement. This rapid approach is likely to be useful for intrinsically disordered proteins, whose repetitive sequence composition and flexible nature can facilitate aggregation.

1. Introduction

Proteolysis commonly occurs during the production and purification of recombinant proteins. Degradation or modification of proteins by proteases during expression and/or purification reduces yield and introduces contaminants similar to the full length protein. For example, proteolytic products that contain a tag or epitope will bind resin as well as the full length protein during affinity purification. For a protease to cut, the protein target site must be embedded in a protein region that is at least 10 amino acids long and unfolded [1]. Some proteins are more susceptible to proteolysis than others, including unstable proteins, proteins with long exposed loops, mutant proteins, molten globules, and intrinsically disordered proteins. A large proportion of gene sequences code for long stretches of amino acids that are likely to be unfolded in solution [2–4]. The occurrence of unstructured regions greater than 40 residues in length is especially common in proteins that regulate critical cell responses [5–10]. Since these regions often play regulatory roles, *in vitro* characterization of the impact of these intrinsically disordered sequences is essential to understand protein function [11–13]. However, intrinsically disordered regions are more readily cut by proteases, and the presence of proteolytic contaminants can alter the apparent function of the purified protein. Because the presence of these contaminating proteolytic products can confound interpretation of downstream characterization assays, removal of these proteolytic products in a rapid and facile manner is crucial.

General approaches to the proteolysis problem include prevention of proteolysis before or during purification and removal of proteolytic products after the initial purification steps. Slowing protein expression can often decrease aggregation and proteolysis. Lowering the incubation temperature after induction, shortening expression time, and/or changing to a less rich media can sometimes minimize or prevent proteolysis before protein purification [14].

A frequently used strategy to prevent proteolysis during purification is to add a variety of protease inhibitors to the extraction buffer. However, this technique is not completely effective for proteins extremely susceptible to proteolysis, such as intrinsically disordered proteins. Finally, if the proteolytic products bind the full-length protein, then they will co-purify with the full-length protein even if they lack affinity tags. An effective method to remove contamination by proteolytic products is to purify under denaturing conditions, yet this harsh method could render the protein of interest non-functional if it is not able to refold properly *in vitro*.

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Existing methods to remove proteolytic products can be slow, providing time for the protein of interest to further degrade and possibly aggregate. In dialysis methods, the full length protein is retained by the membrane, whereas proteolytic products smaller than the pores pass through. This process requires hours to days and thus is not ideal for a protein that continues to degrade or aggregates rapidly [15,16]. Gel filtration chromatography, which separates proteins by size, is also slow, generally taking hours to perform. Affinity chromatography using a tag on the protein or a specific antibody on the resin will capture full-length protein as well as any proteolytic products also containing the tag or epitope. Alternatively, iterative affinity purification using different tags on each of the protein termini can ensure that only full-length protein, which contains both tags, is collected. Although this is an effective method, these fusion tags could potentially alter protein function and/or activity [17,18].

A fast, reliable method is needed to retain protein activity as well as to safely and efficiently remove proteolytic products. In this paper, we demonstrate that two intrinsically disordered proteins generate aggregating proteolytic products that can be removed in minutes by filter flow-through purification. In this rapid technique, full-length protein passes through the filter and aggregated proteolytic products create particles larger than the pores and are thus retained by the filter. Our approach has been demonstrated with two distinct proteins: the putative human lipid-binding protein Sec14-like 1 (Sec14L1), and the *Drosophila* Hox transcription factor, Ultrabithorax (Ubx). Both of these proteins contain intrinsically disordered regions.

2. Results

The first protein tested was the 80 kDa human protein, Sec14L1, in which approximately 32% of the sequence is intrinsically disordered (Fig. 1A). Sec14L1 was expressed in *E. coli* for 48 h at 4 °C after induction with 300 μM IPTG. After centrifugation, cells were lysed using a French press at 1250 psig. After additional centrifugation to remove insoluble material, the supernatant was applied to a Ni^{2+}-binding agarose resin column (Novagen) pre-equilibrated with wash buffer (200 mM NaCl, 50 mM NaH_2PO_4, pH 8.1) and washed for 50 column volumes. Sec14L1 was eluted in wash buffer plus 200 mM imidazole. Gel electrophoresis showed Sec14L1 is on average 57% pure, and western blots identified proteolytic product as the major contaminants (Fig. 2B). Detailed protocols for all methods reported in this paper can be found in Supplemental Material.

To examine if the filter flow-through purification assay would improve purity, we filtered Sec14L1 protein through an Amicon concentrator with 100 nm pores (EMD-Millipore). The supernatant was added to the filter column prior to centrifuging at 10,000×*g* for 10 min at 4 °C. Soluble full length Sec14L1 and a soluble variant (either a long proteolysis product or phosphorylated protein) passed through the filter. However, aggregated proteolytic products were retained by the filter. Quantification of each fraction by western blot demonstrated that soluble Sec14L1 reached approximately 98% purity with no visible contamination from aggregating proteolytic products (Fig. 2B).

To test whether this assay was applicable to other types of proteins or specific to Sec14L1, we examined the 40 kDa *Drosophila* Hox transcription factor, Ultrabithorax (Ubx). Ubx is 53% intrinsically disordered, and contains a predominately structured DNA-binding homeodomain (HD) that binds to the sequence 5′-TAAT-3′ (Fig. 1A) [11,12]. Most of the Ubx sequence is intrinsically disordered, and these regions are extremely susceptible to proteolysis [11]. Since the homeodomain binds the target DNA with higher affinity than the full length Ubx protein, proteolytic contaminants containing this structured domain can out-compete full-length Ubx

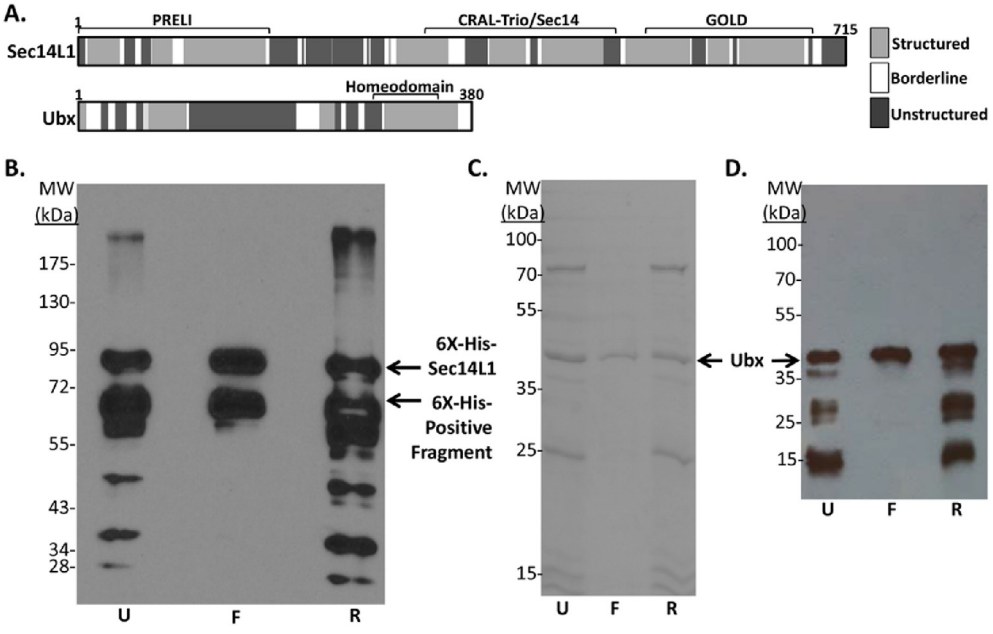

Fig. 1. Filter flow-through purification of the Sec14L1 and Ubx proteins. A) Protein schematics depicting the structured (medium grey, score ≤ 0.4) and disordered (dark grey, score ≥ 0.6) regions of Sec14L1 (top) and Ubx (bottom). White boxes indicate ambiguous regions, in which the score lies between 0.4 and 0.6. Ordered and disordered regions for Sec14L1 were determined from the disorder prediction algorithm PONDR, and scores for Ubx were the average of the IuPred, DisEMBL and PONDR programs as well as native state proteolysis experiments [11]. B) Western blot analysis with Mouse anti-6X His (GeneTex) of a filter flow-through purification of Sec14L1, comparing unfiltered protein (U), the filtrate (F), and retentate (R). Band quantification revealed that the dual Sec14L1 bands were 98% pure, and that 97% of the total Sec14L1 protein was recovered in the filtrate. C,D) SDS-PAGE analysis of filter flow-through purification of Ubx, visualized by coomasie stain (C) and western blot using the anti-Ubx primary antibody FP3.38 (D). Based on band quantification, Ubx purified by filtrate was >90% pure, and 96% of the total full-length Ubx protein was recovered in the filtrate. In both cases, the filter captures aggregated protein fragments, and filtration substantially increases the purity of the protein.