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Purification of protein complexes of defined subunit stoichiometry using a set of orthogonal, tag-cleaving proteases

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ABSTRACT

Tag-free proteins or protein complexes represent certainly the most authentic starting points for functional or structural studies. They can be obtained by conventional multi-step chromatography from native or recombinant tag-free sources. Alternatively, they can be expressed and purified using a cleavable N-terminal affinity tag that is subsequently removed by a site-specific protease. Proteolytic tag-removal can also be performed "on-column". We show here that this not only represents a very efficient workflow, but also drastically improves the purity of the resulting protein preparations. Precondition for effective on-column-cleavage is, however, that the tag-cleaving protease does not bind the stationary phase. We introduce scAtg4 and xlUsp2 as very good and bdSENP1, bdNEDP1 as well as ssNEDP1 as ideal proteases for on-column cleavage at 4 °C. Four of these proteases (bdSENP1, bdNEDP1, scAtg4, xlUsp2) as well as TEV protease display orthogonal, i.e. mutually exclusive cleavage specificities. We combined these features into a streamlined method for the production of highly pure protein complexes: Orthogonal affinity tags and protease recognitions modules are fused to individual subunits. Following co-expression or *in-vitro* complex assembly, consecutive cycles of affinity capture and proteolytic release then select sequentially for the presence of each orthogonally tagged subunit, yielding protein complexes of well-defined subunit stoichiometry.

1. Introduction

Purification of recombinant proteins has been greatly facilitated by the availability of affinity tags mediating specific high-affinity binding to dedicated affinity matrices [1–5]. Such affinity tags may, however, interfere with the function of a protein of interest, influence its structure or preclude crystallization. Therefore, non-tagged proteins are often preferred for functional or structural studies. The classical purification of tag-free proteins using various column chromatographic techniques is often tedious and requires detailed knowledge about the target protein's individual properties. Such purifications can therefore generally not be performed using standardized protocols. In most cases, however, untagged proteins can be produced using an elegant workaround: The target protein is first expressed as a fusion with an affinity tag and a linker presenting a recognition module for a site-specific protease. During or after the purification via the engineered affinity tag, the tag-free target protein is released using the cognate protease ([5,6]).

Such proteolytic removal of affinity tags can be accomplished in solution after elution from the affinity resin. While allowing free access of the protease to its substrate, this procedure has the disadvantage that the cleaved affinity tag has to be separated from the target protein. This generally necessitates a buffer exchange (to remove the prior used eluent) and a "reverse affinity purification step", during which the tag and any non-cleaved fusion protein (still containing the tag) are re-bound to the affinity resin, while the tag-free target protein now remains in the non-bound fraction.

An alternative to such post-elution removal of affinity tags is on-column cleavage. Here, the target protein is released from the affinity resin by directly treating the loaded resin with a specific tag-cleaving protease [7,8]. This method offers several advantages. It not only makes purifications more time-efficient by avoiding any lengthy buffer exchange and reverse chromatography steps but also allows the target proteins to be specifically released from the resin under very mild conditions: As the elution buffer differs from the washing buffer only by a minute amount of protease, on-column cleavage bypasses more drastic elution conditions such as high concentrations of competitor, significant alterations in the buffer composition or pH changes.

Within its cellular context, the physiologically relevant form of a protein is often not a single polypeptide but a complex

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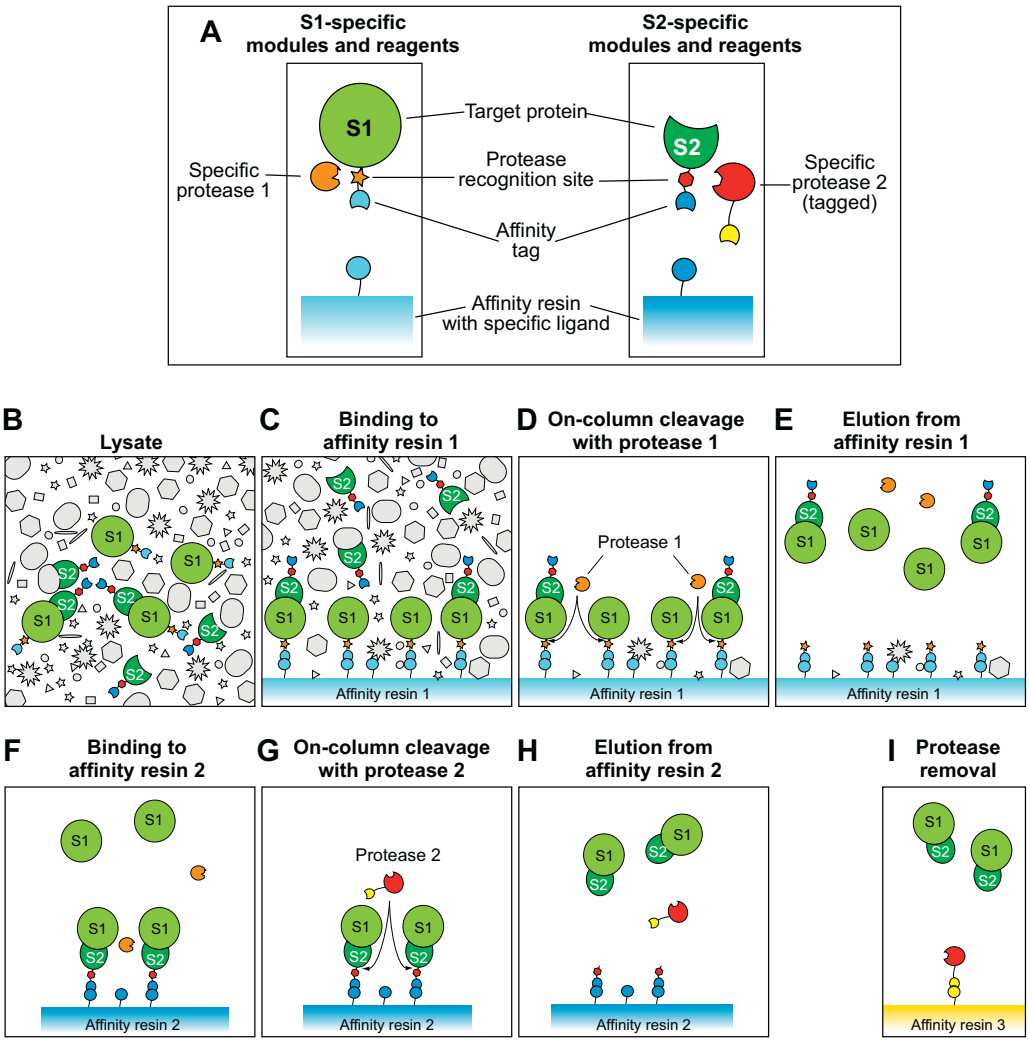

Fig. 1. Purification of a stoichiometric binary complex using two consecutive affinity purification steps with on-column cleavage. (A) Description of components used in the schemes (B)–(I). After co-expressing the two subunits S1 and S2 of the target complex (B), the binary complex is separated from host proteins and individual subunits S1 and S2 by two consecutive affinity chromatography steps using orthogonal tags and protease recognition modules on each of the proteins (C)–(I): The binary complex (and surplus subunit S1) is first bound to affinity resin 1 via the affinity tag on subunit S1 (C). After washing, specifically bound proteins are released from the resin using a site-specific protease recognizing the protease recognition module (D). All contaminant proteins not containing the proper protease recognition module will remain bound to the resin (E). In the second affinity chromatographic step (F)–(H), the binary complex S1•S2 is separated from surplus subunit S1 by binding to affinity resin 2 specifically recognizing the tag fused to component S2 (F) and similarly cleaved off with a component S2-specific protease (G and H). If desired, the protease can be removed e.g. via an adequate affinity resin (I). Protein complexes with more than two subunits can be purified in analogously using an appropriate number of orthogonal affinity matrices and orthogonal protease systems.

comprising two or even multiple subunits. Structural and functional characterization of such protein complexes thus critically relies on purification strategies that allow controlling the stoichiometry of subunits. Provided functional subunits can be produced in the absence of their binding partners, protein complexes can be assembled from individually pre-purified subunits. Alternatively, multiple subunits can be expressed and assembled *in situ* within the same host cell. In both cases, the assembled complex needs to be separated from excess non-assembled subunits and partially assembled sub-complexes. This can be a challenging task. We now propose a general and straightforward strategy for purification of protein complexes with defined subunit stoichiometry that exploits the combined discriminative power of multiple affinity matrices and proteases (Fig. 1). Briefly, by tagging individual subunits of a given protein complex with orthogonal affinity tags and orthogonal protease recognition modules, consecutive sequences of affinity capture and proteolytic release allow selecting for the presence of each tagged subunit individually. This strategy thus provides a streamlined purification scheme and a defined stoichiometry of subunits alongside with a product purity conforming the highest standards. Although Fig. 1 only shows the purification of a binary complex, protein complexes with more than two subunits can be purified in an analogous manner.

Evidently, this strategy requires multiple proteases with orthogonal (i.e. mutually exclusive) specificities. In the accompanying paper [19] we introduced five new site-specific proteases (bdSENP1, bdNEDP1, ssNEDP1, scAtg4, xlUsp2) for tag-cleavage in solution. We show here that scAtg4, xlUsp2, bdSENP1 and bdNEDP1 are indeed fully orthogonal to each other and to TEV protease, even under conditions of an up to 10,000-fold over-digest. We further show that scAtg4, xlUsp2, bdSENP1 and the two NEDP1 proteases perform very well in on-column cleavage, even at the low temperatures (4 °C) that are preferred for gentle protein purification. For example, 30 nM bdSENP1 are sufficient to elute a ≥3000-fold molar excess (≈100 μM) of a His-SUMO-tagged target from a Ni^{2+} chelate matrix within one hour at 4 °C. For comparison, elution of an analogously tagged TEV substrate required a 300-fold higher TEV protease concentration and higher temperatures (≥16 °C). Finally,