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Affitins as robust tailored reagents for affinity chromatography purification of antibodies and non-immunoglobulin proteins

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ABSTRACT

Affinity chromatography is a convenient way of purifying proteins, as a high degree of purity can be reached in one step. The use of tags has greatly contributed to the popularity of this technique. However, the addition of tags may not be desirable or possible for the production of biopharmaceuticals. There is thus a need for tailored artificial affinity ligands. We have developed the use of archaeal extremophilic proteins as scaffolds to generate affinity proteins (Affitins). Here, we explored the potential of Affitins as ligand to design affinity columns. Affitins specific for human immunoglobulin G (hIgG), bacterial PulD protein, and chicken egg lysozyme were immobilized on an agarose matrix. The columns obtained were functional and highly selective for their cognate target, even in the presence of exogenous proteins as found in cell culture media, ascites and bacterial lysates, which result in a high degree of purity (~95%) and recovery (~100%) in a single step. Anti-hIgG Affitin columns withstand repetitive cycles of purification and cleaning-in-place treatments with 0.25 M NaOH as well as Protein A does. High levels of Affitin productions in *Escherichia coli* makes it possible to produce these affinity columns at low cost. Our results validate Affitins as a new class of tailored ligands for the affinity chromatography purification of potentially any proteins of interest including biopharmaceuticals.

1. Introduction

In the manufacture of therapeutic proteins, affinity chromatography contributes significantly to reduce the processing cost as a high degree of purity can be reached in one step. The fusion of proteins to polypeptide tags, such as the hexahistidine tag, is widely used to facilitate protein purification by affinity chromatography [1]. However, this approach is problematic when sequence modifications are neither desirable nor possible.

Thus, specific ligands for proteins of interest can be helpful for affinity purification. For instance, concanavalin A and amylose enable the purification of glycoproteins [2] and maltose binding protein [3], respectively. Among therapeutic proteins, monoclonal antibodies are of great interest, as they accounted for about half of the sales in the European Union and the USA in 2010 [4]. Several bacterial surface proteins have been identified as affinity reagents and are commonly used to purify antibodies or some of their fragments. Binding specificities of these proteins differ between source species and antibody subclasses. For instance, Protein G and Protein A from group G *Streptococci* and *Staphylococcus aureus*, respectively, are able to bind IgG mainly *via* their Fc region [5,6], while Protein L from *Peptostreptococcus magnus* recognizes antibodies through light chain interactions [7]. Protein A strongly binds human IgG1, IgG2 and IgG4 while Protein G strongly binds all human subclasses. Proteins A and G bind rabbit IgG strongly, while Protein L binds them weakly (see [8] for a review).

Thus, depending on the application, the choice of the ligand is critical. A major drawback of these existing binders is that they may not fit specific needs. For non-antibody proteins, the problem is even more serious, as often no natural partner is known with properties suitable for use as an affinity reagent. Therefore, it is worth developing new reagents suitable for affinity chromatography by chemistry or molecular evolution, with specificity and affinity for the protein of interest.

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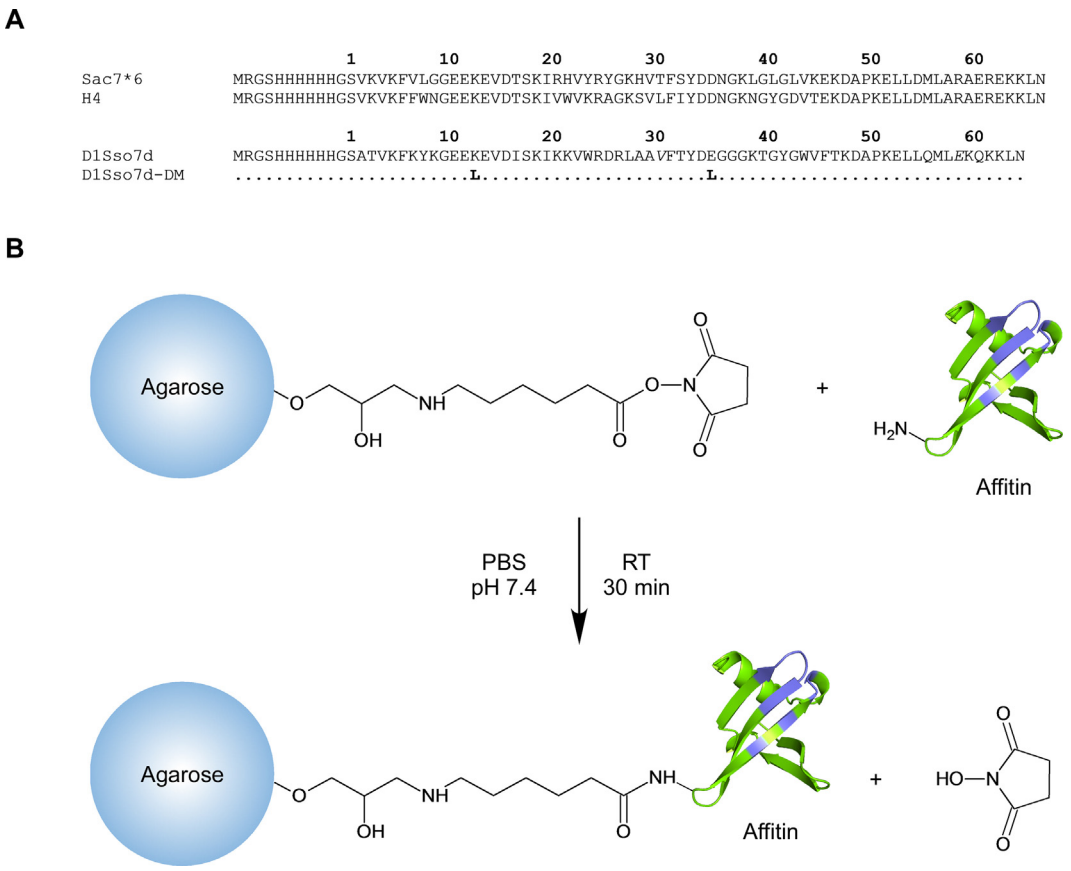

Fig. 1. Sequences of the four Affitins studied and scheme for their coupling to amine-reactive agarose. (A) Sac7*6, and H4 have a specificity for the bacterial PulD-N protein fragment and chicken HEWL, respectively, while D1Sso7d and D1Sso7d-DM have a specificity for hIgGs. Residues common to D1Sso7d and D1Sso7d-DM Affitins are indicated by a dot. (B) Ten milligrams of each Affitin were coupled in PBS *via* their amines to *N*-hydroxysuccinimide-activated agarose matrix to prepare affinity columns. The Affitin in the scheme is a model of the D1Sso7d structure depicted as green ribbons, with residues substituted during the generation of the IgG binding site highlighted in blue [28]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The structure-based design of chemicals and peptidic mimetics have been used to obtain artificial ligands for targets such as antibodies (see [9,10] for reviews), insulin [11], and plasminogen activator [12]. Although some progress has been made in recent years [13,14], obtaining a ligand with a well-defined selectivity is not easy to achieve. Another approach is to develop affinity reagents from proteins, termed alternative scaffold proteins, by combinatorial protein design. For example, this was used to convert an IgG-binding domain from Protein A into an IgA binder [15] and to isolate Fc binders from designed ankyrin repeat protein libraries [16]. Numerous alternative scaffold proteins have been proposed (see Refs. [17] and [18] for reviews), but few of them are really usable for demanding applications, such as affinity chromatography.

Ideally, to be suitable for affinity purification, an affinity reagent should (i) be highly specific for the target to be purified, (ii) show a cost-effective production and (iii) have a high thermal and chemical stability, as this is often associated with a long column life cycle. For use in single-use disposable columns, it is also important that the reagent is at least resistant to the conditions used for the elution of the target. Moreover, to ensure safe reusability of columns, the affinity reagent must be resistant to extreme alkaline pH used for cleaning-in-place (CIP) procedures, which are part of good manufacturing practices (GMP), and for the minimization of product contamination by leached ligand fragments.

We have previously described the use of the small (7 kDa) archaeal hyperthermophilic and acidophilic Sac7d protein and its homologues as a scaffold to design tailored artificial affinity proteins (Affitins) [19,20]. Using combinatorial engineering, we have generated Affitins with dissociation constants in nanomolar and subnanomolar ranges, and with specificity for their targets, such as bacterial protein PulD [19,21,22], chicken egg lysozyme [20,23–26] and human IgGs [27,28]. Recently, we validated the structural basis of two of their modes of binding by solving the structures of three Affitins in complex with their cognate targets [25]. Affitins are chemically and thermally stable (from pH 0 up to 12, toward detergents and chaotropic agents, and up to 90 °C). We have shown that it is possible to further increase the stability of Affitins toward alkaline conditions, up to at least pH 13, *via* a grafting/mutation strategy [28]. Furthermore, we reported that Affitins are overproduced in *E. coli* with yields from several dozen to several hundreds of milligrams per liter of culture. Thus, Affitins exhibit all the desired properties for their use as reagents in affinity chromatography.

Here, for the first time, we present the use of Affitins, covalently immobilized in columns, as reagents capable of selectively capturing three unrelated proteins from heterogeneous protein mixtures: human IgGs, bacterial PulD protein and hen egg white lysozyme (HEWL). Furthermore, to gain an insight into the potential of purification processes using Affitins, we compare several anti-IgG Affitins and Protein A columns to assess their resistance to repetitive CIP procedures using sodium hydroxide. Our results demonstrate the great potential of Affitins as designed ligands for robust affinity chromatography columns.