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High-Yield Production and Cost-Effective Purification of an Alkali-Resistant Hexameric Protein A Variant for Antibody Affinity Chromatography

Carolina Mota Natal[1,2] | Claudia Lacombe[3] | Alexander Zollner[3] | Catarina Domingos[1,2] | José Mendes[4,5] | André Nascimento[4,5] | Ana Margarida Gonçalves Carvalho Dias[1,2] | Arménio Jorge Moura Barbosa[1,2] | Cristina Peixoto[4,5] | Birgit Wiltschi[3] | Alois Jungbauer[3] | Ana Cecília Afonso Roque[1,2]

[1]Associate Laboratory i4HB – Institute for Health and Bioeconomy, Department of Chemistry, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal | [2]UCIBIO – Applied Molecular Biosciences Unit, Department of Chemistry, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal | [3]Institute of Bioprocess Science and Engineering, Department of Biotechnology, BOKU University, Muthgasse, Vienna, Austria | [4]iBET, Instituto De Biologia Experimental e Tecnológica, Apartado, Oeiras, Portugal | [5]ITQB-NOVA, Instituto De Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, Oeiras, Portugal

Correspondence: Birgit Wiltschi (birgit.wiltschi@boku.ac.at) | Ana Cecília Afonso Roque (cecilia.roque@fct.unl.pt)

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ABSTRACT

Recombinant Staphylococcal Protein A is the most widely used affinity ligand for industrial antibody purification, as well as for research and discovery of new antibodies. It has become a platform technology throughout the antibody manufacturing industry for the direct capture of products from a clarified cell culture supernatant. However, protein A adsorbents contribute significantly to manufacturing costs in the early stage of process development. In this work, we aimed to increase the production yield of an engineered protein A ligand comprising six repeats of the alkali-resistant protein A-derived Z domain, with a terminal cysteine residue for adsorbent immobilization (Z6AlkC). We coupled the extracellular production of Z6AlkC via secretion in high-cell-density, carbon-limited bioreactor cultures, with a simple and fast ultradiafiltration step for purification. Our bioprocess achieved 4 g/L of secreted Z6AlkC with an 89% purity, which is an improvement from previous reports that obtained intracellular expression and lower yield. We further purified the Z6AlkC protein by IgG-based affinity chromatography (Z6AlkC-P) and ultradiafiltration (Z6AlkC-F) and used it as an affinity ligand for antibody purification, as a proof of functionality. Both ligands yielded similar purification antibody behavior, showing that high-performance protein A-based ligands can be produced in scalable, high-productivity, and cost-effective methods.

1 | Introduction

Recombinant Staphylococcal Protein A (rSpA) is the best-known and most widely used affinity ligand for industrial antibody production as well as for research and discovery of new antibodies [1]. It has become a platform technology used across the antibody manufacturing industry for direct capture of products from clarified cell culture supernatants [2–7].

Carolina Mota Natal and Claudia Lacombe contributed equally as first co-authors.

Wild-type *Staphylococcus aureus* protein A (SpA) consists of five homologous domains E, D, A, B, and C, and binds the Fc region of antibody molecules with high affinity [8, 9]. As a standard procedure, SpA-affinity columns are regenerated using alkaline conditions because these strip residual proteins, DNA, and lipids after antibody elution. However, SpA has several amino acids that are highly susceptible to degradation by alkaline conditions [10–12]. The regeneration under alkaline conditions, also known as cleaning-in-place (CIP), rapidly reduces the binding capacity of the SpA-affinity chromatography columns. To counteract the denaturing effect of CIP procedures, the B domain was previously mutated and further engineered into the synthetic Z domain, which represents a consensus sequence of all domains present in wild-type protein A and, after protein engineering, can withstand alkaline conditions (Z domain N23T F30A) [13–15]. Multivalent versions by concatenation of several Z-domains were created, resulting in improved SpA-affinity adsorbents further commercialized as MabSelect Sure (commercialized by Cytiva). Multimerization is, in fact, an important aspect observed in engineered SpA-derived purification matrices, as it is possible to create longer and multivalent ligands by increasing the number of Z domains. The binding capacity of multimers consisting of two to nine repeats increased with the number of Z-domain repeats. Maximum binding occurred with eight repeats, while larger concatemers showed lower capacity due to stereochemical hindrance [16]. The latest commercially available affinity adsorbents contain concatemers with six Z-domain repeats [17].

Several engineered SpA variants have been produced recombinantly in safe bacterial hosts such as *Escherichia coli*. The bacterial expression of Z domains and concatenates has been reported in shake-flask cultures, indicating titers of approximately 50 mg/L [18, 19]. Purification is typically performed by IgG-affinity chromatography [19], which greatly increases the purity but also the production costs.

The extracellular production of recombinant proteins by secretion, utilizing the periplasmic space of *E. coli* and a leaky outer membrane to release the protein into the extracellular space, is a production strategy that enables higher productivity and enriches the supernatant in the protein of interest, thus increasing the purity. This strategy enables a less costly purification of Z-domain repeats and contributes to reducing the overall manufacturing cost of antibodies. Still, there are some challenges to overcome in implementing this strategy, related to low production yield when producing longer concatemers and the purification requires expensive IgG-based affinity chromatographic columns [20].

In this work, we developed an easy-to-implement method for the soluble expression and purification of six concatenated Z-domains (Z6AlkC, Figure 1). To construct Z6AlkC, we concatenated six repeats of the alkaline-resistant Z-domain mutant N23T F30A described in Linhult et al. [14] and added an N-terminal PelB sequence for secretion into the medium. We added the C-terminal linker GSCGS for targeted immobilization on chromatography resins by the single cysteine residue. After producing Z6AlkC in a carbon-limited bioreactor culture with fed-batch procedures, we used two independent purification methods to obtain Z6AlkC, namely the standard strategy—IgG-based affinity chromatography—and ultradiafiltration as an alternative, which is less labor intensive and cost-effective. To demonstrate the activity of the purified Z6AlkC ligands, we immobilized them on chromatography beads, showing similar antibody purification performance. Thus, our work establishes a platform for the scalable and productive production of protein A variants as ligands for affinity chromatography.

2 | Materials and Methods

2.1 | Production of Z6AlkC

All buffer ingredients were purchased from Carl Roth (Karlsruhe, Germany). Prior to purification, the buffers were filtered (0.2 μm pore size, Sigma–Aldrich, St. Louis, MO) and degassed.

2.1.1 | Expression Plasmid and Strain

Experiments in this study were performed with *E. coli* BL21(DE3) (genotype: F^- ompT gal dcm lon hsdSB(rB^- mB^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]; Coli Genetic Stock Center #12504). Q5 High-Fidelity DNA polymerase and restriction enzymes were from New England Biolabs (Ipswich, MA, USA) (supplementary information, Figure S1) and were used according to the manufacturer's instructions. The *z6alkC* coding sequence was reverse translated, codon optimized for expression in *E. coli*, and synthesized by Twist Bioscience (San Francisco, US). The linear double-stranded DNA cassette encoding *z6alkC* fused to the *pelB* sequence and the linker sequence was integrated into the pET30a plasmid by restriction cloning, which resulted in plasmid pZ6AlkC. *E. coli* strain NEB5α (New England Biolabs) was used for plasmid amplification, and the *z6alkC* sequence was confirmed by Sanger sequencing (Microsynth, Vienna, Austria).

2.1.2 | Production of Z6AlkC in Shake Flask Culture

BL21(DE3) was transformed with the pZ6AlkC plasmid by electroporation using a 0.1 cm gap electroporation cuvette (Biozym, Vienna, Austria) and the electroporator (BTX, Shelton, CT) set at 1800 V, 200 Ω, and 25 μF. Super optimal broth with catabolite repression medium (SOC medium, Thermo Fisher Scientific, Waltham, MA) was added to the electroporated cells immediately afterwards, and they were incubated for 1 h at 37°C with shaking. The cells were plated on LB agar (Carl Roth GmbH, Karlsruhe, Germany) plates containing 50 mg/L kanamycin and incubated overnight at 37°C. Master cell banks were prepared using M9ZB medium. Working cell banks were prepared using semi-synthetic medium with glucose (SSM). The ingredients of the culture media SSM and M9ZB were previously described [21, 22].

To start a shake flask culture, SSM was inoculated with the BL21(DE3){pZ6AlkC} strain to an attenuance at 600 nm (D_{600}) of 0.1 in a baffled shake flask. The culture was incubated at 37°C in an orbital shaker at 200 rpm. 1 mM IPTG was used for induction of gene expression at D_{600} of 0.7, and the production of Z6AlkC occurred at 30°C overnight. Cells were harvested by low-speed centrifugation (10.000 × *g*, 10 min, 4°C), and the cell pellet as well as the media supernatant were frozen at −20°C until further analysis.

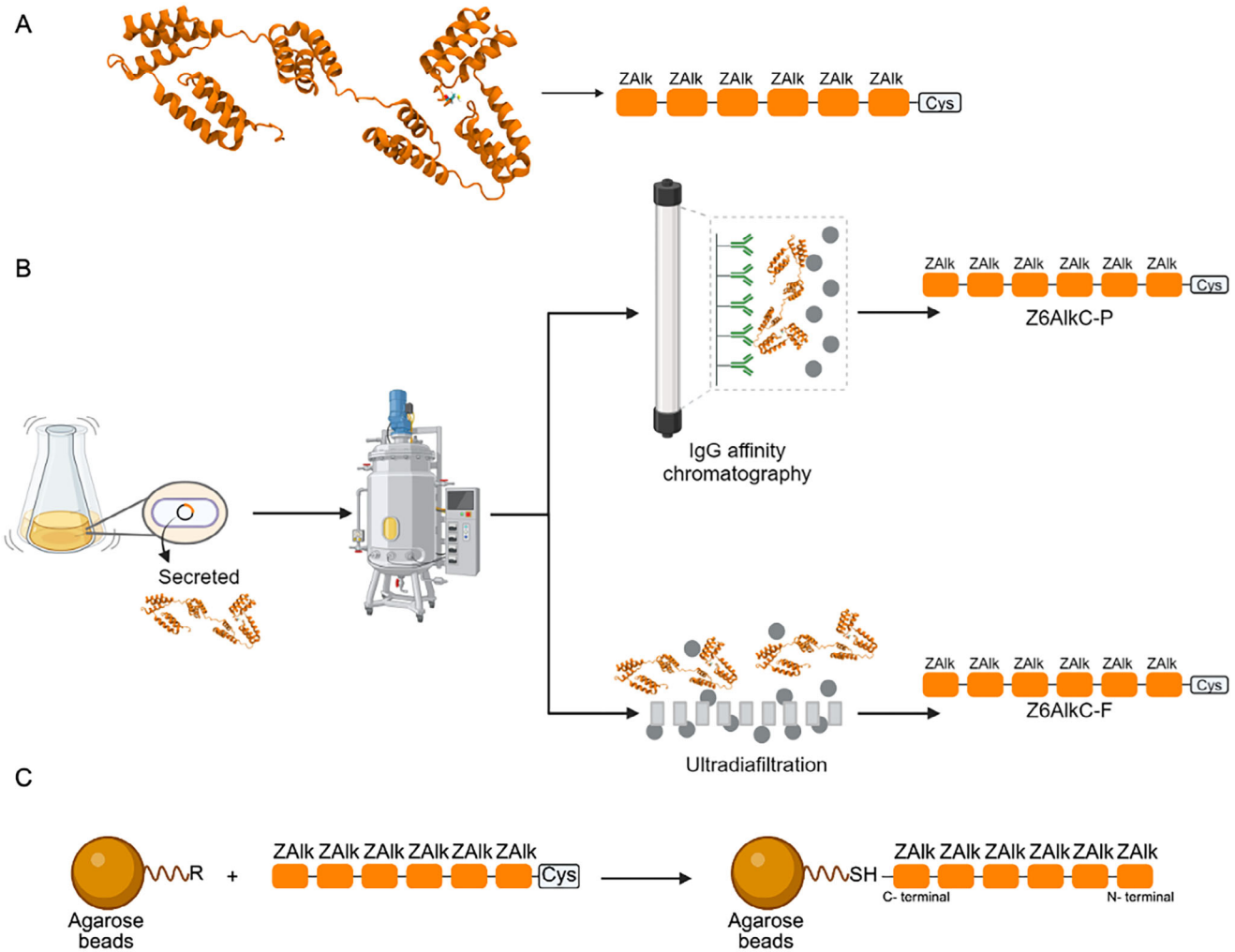

FIGURE 1 | Research strategy developed in this work. (A) Head-to-tail concatenation of six Z domains and C-terminal fusion to a tag containing a single cysteine residue. (B) Production of Z6AlkC in fed-batch bioreactor culture and purification by IgG affinity chromatography (Z6AlkC-P) or ultradiafiltration (Z6AlkC-F). (C) Immobilization of Z6AlkC on the chromatography matrix (two different commercial resins were tested: SulfoLink Coupling Resin and Toyopearl AF-Epoxy-650 M beads).

2.1.3 | Fed-Batch Bioreactor Culture

The fermentation cultures (batch and subsequent fed-batch) were carried out as described previously by Fink et al. [23] in Dasgip Parallel Bioreactor Systems (Eppendorf, Hamburg, Germany) with a working volume up to 1.2 L at the end of the fermentation culture. All media components were purchased from Carl Roth (Karlsruhe, Germany) unless otherwise indicated. A pre-culture of BL21(DE3){pZ6AlkC} strain was grown in SSM containing 50 mg/L kanamycin in a baffled shake flask at 37°C and 200 rpm shaking. To start the batch fermentation culture, 650 mL were inoculated with 25 D_{600} units of the pre-culture. Kanamycin was not added to the batch culture medium. The medium composition is listed in Tables S1–S3. To monitor cell growth, the D_{600} was recorded using a DiluPhotometer (Implen, Munich, Germany) spectrophotometer. The cell density was calculated from the cell dry mass (CDM) by collecting 1 mL culture supernatant in 2 mL Eppendorf tubes (16.000 × g, 10 min, 4°C). The tubes were previously dried at 105°C for 48 h and desiccated for 4 h before weighing. Cell pellets were washed with 1.8 mL dH_2O and centrifuged again (16.000 × g, 10 min, 4°C) and resuspended in 1.6 mL dH_2O. The Eppendorf tubes were weighed again after drying for 48 h at 105°C and desiccated for 4 h. The batch phase occurred at 37 ± 0.2°C and pH 7 ± 0.2 until the CDM reached 10 g/L. During the fed-batch phase, the cells were grown exponentially at a growth rate (μ) of 0.1 h^{-1} for 16 h until a calculated cell density of 29 g/L CDM was reached at the end of fermentation. Gene expression was induced 3 h post-start of the exponential feed phase using 1 mM IPTG at 30 ± 0.2°C. At the end of fermentation, the cell broth was harvested, cells were separated from the medium supernatant by centrifugation (10,000 × g, 30 min, 4°C), and both fractions were frozen at −20°C until further use.

2.1.4 | Product Analysis

The intracellular product analysis was carried out according to Fink et al. for both soluble and insoluble protein fractions [24]. The protein production was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 4%–12% Bis-Tris gels (Thermo Fisher Scientific) if not indicated differently. A mix of 65% sample,

25% 4x NuPAGE LDS Sample Buffer, and 10% 10× NuPAGE Reducing Agent (both Thermo Fisher Scientific) was prepared, and 15 µL were loaded per well. The mix was heated at 95°C for 10 min prior to loading. The gel was stained using Coomassie R250. The Z6AlkC titer was estimated by densitometry analysis using ImageJ-Fiji (version v1-54i) with bovine serum albumin (Sigma–Aldrich, St. Louis, MO) as the quantification standard.

2.2 | Purification of Z6AlkC

Z6AlkC was purified by chromatography using IgG Sepharose 6 Fast Flow resin (Z6AlkC-P) or by a single ultradiafiltration step (Z6AlkC-F). Product analysis occurred as described in Section 2.1.4.

2.2.1 | Chromatographic Purification of Z6AlkC Using IgG Sepharose 6 Fast Flow

All chromatographic purifications of Z6AlkC were performed on an ÄKTA Pure 25 chromatography system (Cytiva, Marlborough, Massachusetts, USA). Buffers were filtered (0.2 µm pore size, Sigma–Aldrich) and degassed prior to use. 25 mL of the resin IgG Sepharose 6 Fast Flow (Cytiva, Marlborough, Massachusetts, USA) was packed into a HiScale 16/20 column (Cytiva, Marlborough, Massachusetts, USA). The purification was performed at a flow rate of 5 mL/min, resulting in a residence time of 5 min. For equilibration, two column volumes (CV) of 0.5 M CH_3COOH (adjusted to pH 3.4 with CH_3COONH_4 (NH_4Ac)) and 2 CV of Tris-saline Tween 20 (TST, 50 mM Tris buffer pH 7.6, 150 mM NaCl, and 0.05% Tween 20) were used. 9 mL of 0.2 µm-filtered culture medium from the fed-batch bioreactor culture was then loaded onto the column. The column was washed with 4 CV of TST and 2 CV of 5 mM NH_4Ac, pH 5.0. Proteins were eluted using 2 CV of 0.5 M CH_3COOH, pH 3.4, and peak fractionation by UV detection was applied. After elution, the column was re-equilibrated with 4 CV of TST. 1 M Tris base was added to the elution fractions containing protein to immediately raise the pH to neutral. To purify all products of the fed-batch fermentation, the chromatography run was repeated 10 times, and the respective elution fractions were pooled.

The total process time was calculated using the following formula (Equation 1):

$$process\ time_{chromatography}\ [min] = \sum CV * residence\ time\ [min] * \sum runs$$

The identity and secondary structure of Z6AlkC-P, as well as its binding affinity toward human IgG, were determined using mass spectrometry (MS), circular dichroism (CD), and microscale thermophoresis (MST). Further details can be found in the supplementary information.

2.2.2 | Purification of Z6AlkC by Ultradiafiltration

The supernatant of the fed-batch bioreactor culture was thawed and centrifuged at 10,000 × *g* for 30 min at 4°C using a Sorvall Lynx 6000 centrifuge (Thermo Fisher Scientific). The supernatant was subsequently filtered using a Bricap C03 Capsule filter (Purcise SLE 0.8/0.2 µm; Cobetter, Hangzhou, China). The material was concentrated 6-fold and buffer-exchanged with 10 times the volume of the reconcentrated volume against phosphate-buffered saline (PBS, ThermoFisher Scientific, Germany) using a Pellicon 2 Mini Cassette with Biomax 10 kDa membrane, A screen, 0.1 m^2 (P2B010A01, Merck, Darmstadt, Germany) at a permeate flux of 46.5 mL/min. The ultradiafiltration was performed on an ÄKTA flux chromatography system (Cytiva, Marlborough, MA, USA). A total of 2 L of culture medium was ultradiafiltered. Permeate fractions of 500 mL each were collected, and 340 mL of retentate was recovered. The membrane was washed with 250 mL PBS to recover residual Z6AlkC. For storage, the membrane was washed using 0.5 M NaCl and stored in 20% (v/v) ethanol. The retentate was filtered again using 0.2 µm Nalgene Rapid-Flow Sterile Disposable Filter Units with PES (ThermoFisher Scientific, Germany). The retentate was aliquoted and stored at −20°C until further use.

The total process time was calculated using the following formula (Equation 2):

$$process\ time_{ultrafiltration}\ [min] = \frac{V_{start}\ [mL] - V_{final}\ [mL]}{permeate\ flux\ \left[\frac{mL}{min}\right]} + \frac{V_{final}\ [mL] * \sum cycles}{permeate\ flux\ \left[\frac{mL}{min}\right]}$$

V_{start}… Volume at the beginning of ultradiafiltration

V_{finalc}… Volume after the concentration step and at the beginning of the buffer exchange step

$\sum$ cycles… sum of buffer exchange cycles

2.3 | Selection of Z6AlkC Sample for Immobilization on Chromatographic Beads

Z6AlkC-P (Z6AlkC purified on IgG Sepharose 6 Fast Flow) and Z6AlkC-F (Z6AlkC purified by ultradiafiltration) (7–9 nmol) were covalently immobilized to 1 mL of SulfoLink Coupling Resin (Thermo Scientific, 20401) through the free C-terminal cysteine according to the manufacturer's instructions. The reactions were performed in triplicate at room temperature. The coupling was done in 50 mM Tris, 5 mM EDTA-Na, pH 8.5. Prior to coupling, tris(2-carboxyethyl)phosphine (Bond-Breaker TCEP Solution, Neutral pH, Thermo Scientific, cat#77720) was added to the Z6AlkC protein to make sulfhydryl groups available for coupling. After coupling, a solution of 50 mM L-Cysteine was used to block any remaining functional groups on the resin. The functionalized resins with Z6AlkC were stored in 20% ethanol at 4°C. Coupling efficiency was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4%–15% acrylamide gradient Tris-glycine precast gels (NZYtech, Lisbon, Portugal, cat# MB46601) at 120 V by loading 15 µL of each sample/well. SimplyBlue SafeStain (ThermoFisher Scientific, Germany) was used for staining, according to the supplier's protocol. The intensity of the band of Z6AlkC in each sample

(load and noncoupled protein—flow-through and washes) was used to quantify the immobilization yield against a calibration curve made with commercial recombinant protein A (SpA, SinoBiological, Eschborn, Germany, cat#10600-P07E). Images were acquired with the Gel Doc XR + System with Image Lab software from Bio-Rad.

Z6AlkC resins (Z6AlkC-P and Z6AlkC-F; 1 g wet weight; 1 g/mL slurry) were packed by gravity in polypropylene columns (0.8 cm x 6.5 cm; Varian, Lisbon, Portugal). The resins were equilibrated with 10 column volumes (CV) of the binding buffer (PBS pH 7.4, 10 mM sodium phosphate, 1.8 mM potassium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride). The resins were loaded with 1 mL of pure human immunoglobulin G (hIgG, Gammanorm, Octapharma; 10 mg/mL) or with pure Trastuzumab, kindly provided by BOKU (5 mg/mL) solutions in binding buffer. Samples were incubated for 1 h, under orbital agitation, at room temperature. The resin was left to settle by gravity, and then washed with 4 CV of binding buffer, followed by 5×0.5 mL of elution buffer (0.5 M CH3COOH pH 3.4). The first CV elution was incubated for 5 min, without agitation. The eluted fractions were further neutralized with 1 M Tris-HCl, pH 9.5. CIP was performed by applying twice 2 CV of 0.1 M NaOH, followed by 2 CV of distilled water. Assays were performed in triplicate. To quantify the amount of antibody in the samples, the absorbance at 280 nm was measured in a Microplate Reader TECAN Infinite 200 using a 96-well UV-STAR Microplate (Greiner, 675801). The percentage (%) of recovery was calculated based on the following formula (Equation 3):

$$Recovery \quad (\%) = \frac{\text{mol target eluted}}{\text{mol target bound}} \times 100$$

Z6AlkC-F resin (low ligand density) was tested in the AKTA system, and details can be found in the Supporting Information.

2.4 | Z6AlkC-F Immobilized in SulfoLink Coupling Resins

Ligand density optimization: Different concentrations of Z6AlkC-F (0.8, 2.7, 6.7, and 14.0 mg/mL packed bed) were covalently immobilized to 0.2 mL of the commercial resin SulfoLink Coupling Resin (Thermo Scientific, 20401), following the protocol described in Section 2.3. To estimate the immobilization efficiency, total protein concentrations in the load, flow-through, and washes were quantified by Pierce BCA Protein Assay Kits (Thermo Fisher, 23225), according to the supplier's protocol. The standard curve was prepared with Bovine Serum Albumin (BSA) protein standard. The working range of the standard curve was between 25 and 2000 µg/mL. All samples were prepared in triplicate in a Flat Transparent 96-well microplate from Sarstedt. Before absorbance measurements, 25 µL of each sample was mixed with 200 µL of BCA working reagent (50 parts of Reagent A with 1 part of Reagent B) and incubated at 37°C for 30 min. The absorbance at 562 nm was obtained in the Microplate Reader TECAN SPARK. The purity of Z6AlkC was estimated by densitometry from SDS-page gels. 10 µg of total protein was loaded in Tris-Glycine Precast gels 4%–15% acrylamide gradient (NZYtech, MB46601) at 120 V for 1 h 30 min. Images were acquired with Gel DocTM XR + System with Image LabTM software from Bio-Rad.

Binding and recovery with pure hIgG: The Z6AlkC-F resins were equilibrated with 10 column volume (CV) of the binding buffer (PBS pH 7.4, 10 mM sodium phosphate, 1.8 mM potassium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride) and 100 µL of 50% slurry (50 µg of wet resin) were transferred to a 96-well filter plate (Agilent, A596002000). The resins were packed by centrifugation (30 s, 2000 rpm), in triplicate. The resins were loaded with 200 µL of pure human immunoglobulin G (hIgG, Gammanorm, Octapharma; 10 mg/mL) in binding buffer. Samples were incubated for 1 h, with agitation, at room temperature. After the incubation, the resins were centrifuged for 30 s, 2000 rpm, and washed with 4 × 200 µL of binding buffer, followed by 5 × 200 µL of elution buffer (0.5 M acetic acid, pH 3.4). The first elution was incubated for 5 min, without agitation. Between each wash and elution, the resins were centrifuged for 30 s, 2000 rpm. After the screening with human IgG, the resins were washed with 10 CV of binding buffer and stored in 20% ethanol at 4°C. To quantify the amount of antibody in the samples, the absorbance at 280 nm was measured in a Microplate Reader TECAN SPARK using a 96-well UV-STAR Microplate (Greiner, 675801). Equation 3 was used to calculate the percentage (%) of recovery. The data was processed by calculating the mean values and standard deviation in OriginPro 2026 software. Furthermore, the data were further statistically characterized to determine the statistical variance of binding and elution values. For that, the pair-wise comparison with the Tukey test was used and considered a variance significance below 0.05 (for p-values < 0.05, data were considered different). The graph's data was plotted using the Paired Comparison plot app available in OriginPro2026 software.

Purification of mAbs using Z6AlkC-F ligand: 3 mL of commercial resin SulfoLink Coupling Resin (Thermo Scientific, 20401) was modified with Z6AlkC-F (ratio 3 mg ligand/mL resin) using the same protocol described above. The resin (1 mL) was packed into a Tricorn 10/20 column (Cytiva), according to the manufacturer's instructions. All the chromatographic runs were performed in an ÄKTA Pure 150 (Cytiva, Uppsala, Sweden), with UV, pH, and conductivity constantly being monitored. The buffers applied for all runs were PBS, pH 7.4, as the binding buffer, and acetic acid, 0.5 M, pH 3.4, as the elution buffer. In this work, we used two different human IgG1: Mab1 and Mab2. Mab1 is constitutively produced in Chinese hamster ovary (CHO) cells, clarified by centrifugation and 0.22 µm sterile filtered, purified by Protein A affinity chromatography, and diluted in equilibration buffer (PBS, pH 7.4). Mab2, as Mab1 is constitutively produced in CHO cells, is also clarified by centrifugation and 0.22 µm sterile filtered, but without any further purification step. The $DBC_{10\%}$ assay was performed to obtain a full breakthrough curve using Mab1. Following that, 4.3 mL Mab1 (2 mg/mL) was loaded. Finally, 20 mL of Mab2 was loaded. In all the runs, the same method and buffers were applied. Before load, the column was equilibrated with binding buffer, and after load, a 5 CV wash step was applied, followed by a 5 CV elution step, and finally, the column was CIP with 0.1 M NaOH. Once the CIP was finalized, the column was re-equilibrated with binding buffer. In all the steps of the run, a residence time of 3 min was used (including $DBC_{10\%}$). For all runs, flow-through (FT) and elution samples (E) were collected and

pooled together. The collected samples were analyzed by SDS-PAGE gel by mixing 13 µL of each sample with the loading buffer (LDS sample buffer 4×, Thermo Fisher Scientific, Waltham, MA, USA) and sample reducing agent 10× (Invitrogen, ref. NP004, Waltham, MA, USA). Samples were denaturized at 99°C for 5 min. Proteins were separated under reducing conditions in a 4%–12% (w/v) polyacrylamide NuPAGE gradient precast gel (Thermo Fischer Scientific). Samples were resolved for 60 min using NuPAGE MOPS SDS running buffer at a constant voltage of 200 V and stained with Sypro Ruby Pro (Thermo Fischer Scientific) overnight, following the manufacturer's protocol. Relative quantification of the antibody was performed by HPLC. A Waters XBridge Protein BEH SEC Column, 200Å, 3.5 µm, 7.8 × 150 mm, Hybrid, Usp (Waters, Milford, MA, USA) size exclusion column was used to evaluate the percentage of antibody in flow-through and elution samples. PBS was used as the mobile phase.

2.5 | Z6AlkC-F Immobilized in Toyopearl AF-Epoxy-650 M Beads

For the immobilization of Z6AlkC-F on Toyopearl AF-Epoxy-650 M beads (Tosoh Bioscience, Tokyo, Japan), a protocol based on Von Roman et al. [25] and Padwal et al. [26] was adapted. 1 g of dry resin was mixed with 8 mL of NaH_2PO_4 buffer (1.5 M, pH 6.5). Swelling was allowed for 1 h and resulted in 4.1 mL of settled resin. By removing supernatant, the solution was reduced to a 75% slurry (5.3 mL). The Z6AlkC-F was diluted in the NaH_2PO_4 buffer to a concentration of 0.3 mg/mL. 16.67 mL of this dilution was placed on the 75% slurry resin. Incubation took place for 16 h at 28°C on a shaker. After incubation, the supernatant was removed, and for blocking, 10 mL of Tris buffer (1 M, pH 9.0) was added, followed by incubation for 1 h at 28°C. After this step, the resin was washed with 10 mL of PBS buffer (pH 7.4) three times. To estimate the immobilization efficiency, the same protocol described in Section 2.4. With the resulting resin, a 2.67 mL resin in a Tricorn 10 column (Cytiva, Uppsala, Sweden) was packed and used for the purification runs of purified and crude IgG1 (Trastuzumab) material kindly provided by Gabriele Recanati (BOKU). All purification runs were performed on an ÄKTA Pure 25 instrument (Cytiva, Uppsala, Sweden) using Buffer A (PBS, pH 7.4) and Buffer B (0.5 M acetic acid, pH 3.4). To evaluate both binding performance and chemical stability, the runs were conducted in the following sequence: (1) a preliminary full breakthrough curve to determine the dynamic binding capacity (DBC); (2) a 10% breakthrough ($DBC_{10\%}$) run using 10.6 mL purified IgG (1.71 mg/mL); (3) a Cleaning-in-Place (CIP) step consisting of a 30-min hold with 0.1 M NaOH to assess ligand stability; (4) a repeat of the $DBC_{10\%}$ run with purified IgG to verify capacity retention; and (5) a purification run using 60.0 mL of crude (after centrifugation and a 0.2 µm filtration) IgG-containing CHO cell culture supernatant (0.34 mg/mL, corresponding to a total load of 20.6 mg). Before the first run, the column was equilibrated with Buffer A for 5 CV. After the respective sample load, a wash step with 6.71 CV of Buffer A was applied, and elution was performed with Buffer B for 5 CV. The elution peaks were immediately neutralized with 1 mL of 1 M Tris (pH 9.0). Between all individual runs, the column was re-equilibrated with 10 CV of Buffer A to restore the starting pH of 7.4. During load, wash, and elution, a residence time of 3 min was used. Respective fractions were collected, and IgG concentrations were measured via an analytical Protein A HPLC method as used by Recanati et al. [27]. To prepare a control, 300 µL Toyopearl AF-Epoxy-650 M beads were incubated with 800 µL of 1 M Tris, pH 9.0, for 1 h at 28°C. The resin was then washed three times with 800 µL of PBS, pH 7.4. IgG binding assays were performed in duplicate by incubating 50 µL of resin with 2 mL of a 1 mg/mL IgG solution (2 mg of IgG) overnight at 10 rpm.

3 | Results and Discussion

3.1 | Comparison of Different Production Scales and Purification Methods for Z6AlkC

Increased recombinant staphylococcal Protein A (rSpA) titers, design of robust rSpA variants, and an increased capacity of rSpA resins—for instance, by controlled immobilization and expanded ligand avidity—are main efforts to deliver high-performance affinity resins for mAb purification [28, 29]. To match these industrial needs and research efforts, we set out to produce an alkali-resistant rSpA variant at high levels. The Z domain mutant N23T F30A previously described by Linhult et al. [14] is resistant to the alkali-treatment during CIP procedures in industrial production. We fused six Z N23T F30A domains in a head-to-tail fashion (Figure 1A) to increase the avidity of the ligand [14, 18]. To reduce sequence repetition on the DNA level, we reverse-translated the amino acid sequence of the Z N23T F30A domain (Table S4) into six distinct coding sequences for the ZAlk monomer using the codon scrambler software [30]. The sequence of the hexaconcatemeric Z6Alk was then codon optimized for expression in *E. coli*. We did not include a polyhistidine fusion tag to avoid decreased expression levels and nonspecific binding of host cell proteins during mAb purification [28]. Furthermore, we fused the PelB secretion signal to the N-terminus of the hexaconcatemer to secrete the protein into the growth medium for facilitated purification. Secretion would reduce the purification costs because no cell lysis steps were required [2, 31]. In addition, we anticipated that the secretion would reduce the risk of contamination of the ligand preparation with host cell proteins and impurities such as endotoxins [28]. A C-terminally fused GSCGS linker sequence introduced a single cysteine residue into the protein, which we termed Z6AlkC. This reactive handle would allow directed immobilization on a chromatography resin (Figure 1B). The production of our untagged, secreted Z6AlkC differs noticeably from commercial rSpA, which is produced intracellularly in *E.coli* BL21(DE3), cell-lysed and purified via its polyhistidine fusion tag [28].

First, we produced Z6AlkC in shake-flask cultures of the *E. coli* host BL21(DE3). During 21 h of induction, we reached a final D_{600} of 8.9, which corresponded to a cell dry mass (CDM) of 2.5 g/L estimated from the D_{600} measurement. The titer was 250 mg/L secreted Z6AlkC (corresponding to 100 mg/g CDM) and 200 mg/L of intracellularly produced soluble Z6AlkC (corresponding to 70 mg/g CDM) calculated from SDS-PAGE densitometry analysis (Figure 2A). We calculated the purity by the ratio of the densitometry of the Z6AlkC band relative to the total of host cell protein bands. It was estimated that the Z6AlkC purity is 28 and 92% in the intracellular and in the secreted fractions when produced in the shake flask cultures. We noticed the presence of a second band below the expected band of Z6AlkC, which was

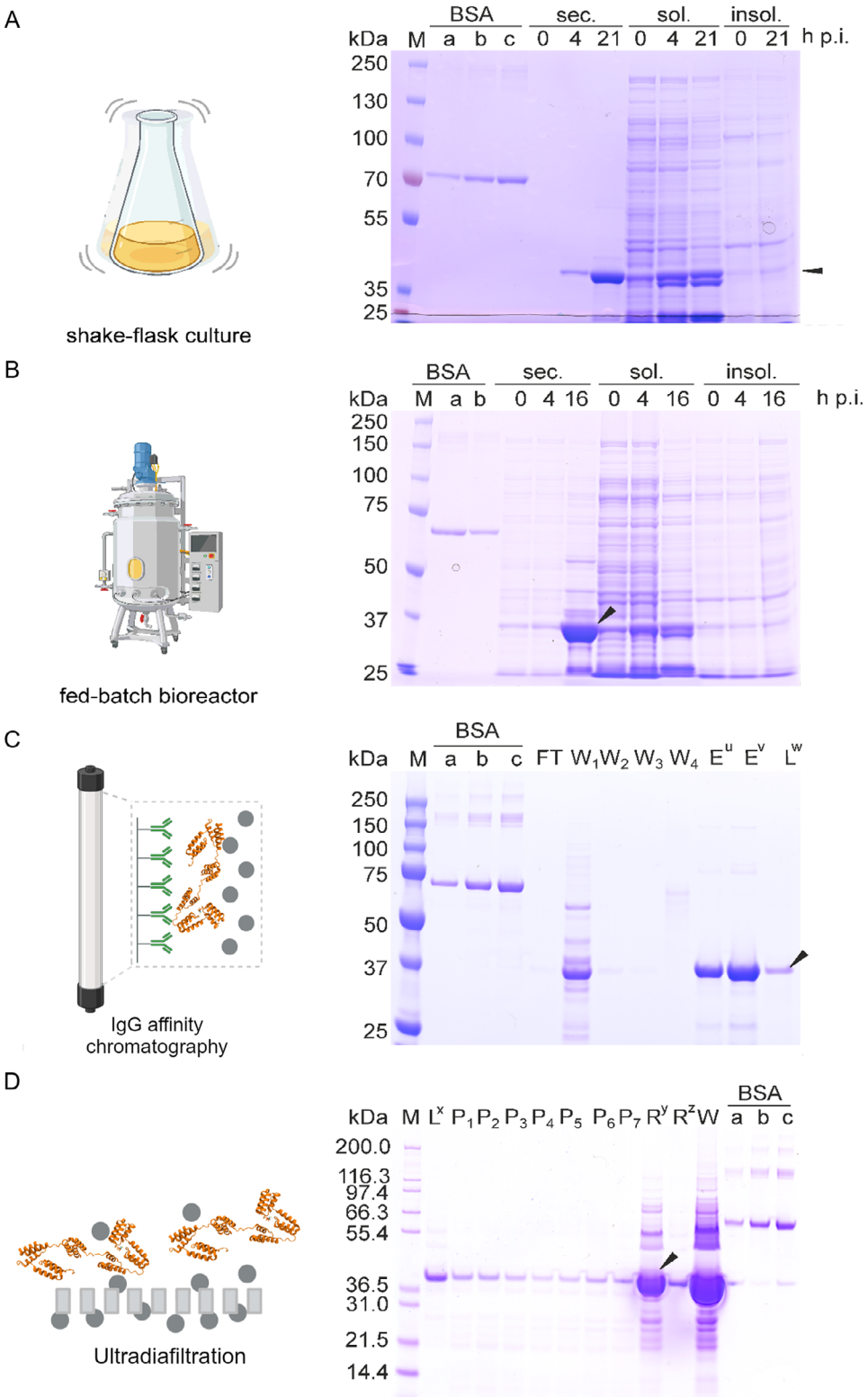

FIGURE 2 | Strategies used for the production of Z6AlkC. Z6AlkC was produced in shake flask culture (A) and fed-batch bioreactor culture (B). The secreted (sec.), soluble (sol.), and insoluble (insol.) protein fractions of both production processes were analyzed by SDS-PAGE. Z6AlkC was then purified by immunoglobulin G (IgG) affinity chromatography (C) and ultradiafiltration (D). Z6AlkC (calculated molecular weight (MW_{calc}) 39 kDa) is indicated by the black arrow. BSA, bovine serum albumin, was loaded at 0.24 (a), 0.49 (b), and 0.73 (c) µg per lane for protein quantification. M, molecular size marker; the bands of the marker are indicated on the left gel margins; L, load; FT, flow-through; W, wash fractions; E, elution; P1-7, permeates; R, retentate. The respective dilutions are indicated by the numbers below the letters. Coomassie-stained 4–12% Bis-Tris gels are shown. The expression of Z6AlkC protein was analyzed at 0, 4, or 21 h post-induction (p.i.) of Z6AlkC. The dilutions are indicated by the letters E^u – 13-fold dilution; E^v – 4-fold dilution; E^w – 125-fold dilution; L^x – 50-fold dilution; R^y – 17-fold dilution; R^z – 833-fold dilution.

a degradation product of Z6AlkC as confirmed by mass analysis (data not shown). Thus, approximately 60% of the protein was found in the secreted fraction.

To increase the amount of Z6AlkC for the subsequent purification, we scaled the production up to a 1 L bioreactor. The *E. coli* strain BL21(DE3) carrying the expression construct for Z6AlkC was cultivated in a fed-batch bioprocess. The batch process was designed to yield a cell density of 10 g/L CDM. Nine hours after inoculation, the cells had depleted the glucose in the batch medium, and the exponential feed was started (Figure S2A). During the fed-batch phase, the cells were supplemented exponentially with feed medium to maintain a growth rate of $\mu = 0.1\ h^{-1}$. The production of Z6AlkC was induced with 1 mM IPTG 3 h into the fed-batch phase, and protein production was allowed to proceed for 16 h at 30°C. The exponential feed phase was designed to reach a cell density of 29 g/L until the end of fermentation. The CDM in the bioreactor culture was 11-times higher compared to the shake flask culture. Indeed, we measured a CDM of 27 g/L at the end of the fermentation. The CDM measurements toward the end of the fermentation were lower than calculated, which suggests that cell lysis occurred (Figure S2B). We produced 4 g/L (corresponds to 150 mg/g CDM) secreted Z6AlkC in the fed-batch bioreactor culture plus 3 g/L (100 mg/g CDM) of intracellularly produced soluble Z6AlkC C (Figure 2B). The Z6AlkC did not occur in the insoluble protein fraction. Of a total of 7 g/L (or 250 mg/g CDM) produced Z6AlkC, 60% appeared in secreted form in the growth medium. The purity of the proteins was estimated by SDS-PAGE, and the intracellularly produced soluble Z6AlkC and the Z6AlkC secreted fraction were 24% and 89%, respectively. These results correlate with the findings described above in shake flask culture. Linhult [14] reported the production of Z domain variants monomers intracellularly in *E. coli* (50 mg/L). Due to the high purity, the secreted fraction appeared very advantageous in comparison to the intracellular fraction for downstream application. We report here a high cell density production bioprocess of alkali-resistant Z6AlkC in carbon-limited fed-batch culture, which yielded 4 g/L of soluble, secreted Z6AlkC.

The Z6AlkC from bioreactor culture supernatant was purified in two independent ways, either by IgG affinity chromatography (Figure 2C) or by ultradiafiltration (Figure 2D). For the chromatographic purification of Z6AlkC, the harvested material was filtered through a 0.2 μm filtration membrane for the removal of cell debris and other large impurities. Then, it was loaded onto a lgG Sepharose 6 Fast Flow column, followed by a wash step and elution at low pH. The concentration of the eluted material was estimated by gel densitometry analysis as 1.7 ± 0.5 g/L. A purification yield of 98%, due to minor losses during flow-through and wash, with a purity of 93% was achieved as assessed by SDS-PAGE analysis (Figure 2C).

For the purification by ultradiafiltration, the concentration of the product in the retentate was 48.8 g/L as estimated by gel densitometry analysis. Only 1% of the protein was found in the combined permeate fractions, resulting in a purification yield of 99% and a purity of 89%. The results of the purification of Z6AlkC by IgG affinity chromatography (Z6AlkC-P) and ultradiafiltration (Z6AlkC-F) are summarized in Table 1. Yield and purity of both purification processes are comparable to reports in the literature, for example, SpA purification performed by tangential flow filtration and ion exchange chromatography, with a final purification yield of 90% and a purity of 96% [32]. Our approach outperformed ion exchange chromatography in terms of yield reported by Choudhury et al. [33] (yield of 70% and purity >90%). When comparing our two applied methods, ultradiafiltration is clearly to be favoured in terms of processed volume per time. When aiming for increased purity, chromatography should be the method of choice. Notably, the bioprocess we describe here has not yet been optimized. *E. coli* naturally secretes only a few proteins [34]. The impurities in the diafiltrated product, therefore, were most likely host cell proteins that had accumulated due to cell lysis during fermentation. An optimized bioprocess would aim to reduce cell lysis and thus contamination of the secreted product with host cell proteins. Previous work demonstrated that cell lysis can be limited to approximately 1%, while high extracellular titers are maintained [35]. To further reduce impurities, anion-exchange chromatography could serve as a polishing step to remove host-cell proteins, nucleic acids, endotoxins, and lipids.

TABLE 1 | Summary of Z6AlkC preparation from clarified *E. coli* supernatant.

Z6AlkC preparation	Z6AlkC-P (Purified by IgG Sepharose)	Z6AlkC-F (Ultradia-filtrated)
Yield (%)	98	99
Purity (%)	93	89
Final concentration (mg/mL)	1.7	48.8
Volume processed (mL)	90	2000
Process time (min)	815	108
Adsorbent Cost* (€)	660	1540
Water consumed (mL)	4000	250
Volume processed/Process time (mL/min)	0.1	18.5
Volume processed/Adsorbent cost (€/mL)	0.1	1.3
Productivity (g/L/h)	0.1	27.1

**Note*: Costs were estimated based on the materials used, excluding costs of buffers and reagents.

Z6AlkC-P secondary structure was characterized by Circular Dichroism (CD), and it showed the characteristic peaks for α-helix (negative peaks at 208 and 222 nm) that are the known secondary structure of Z-domain (compact folding with 3 α-helixes [36] (Figure S3A), also confirmed by approximately 90% of α-helix, as predicted by BeStSel software. A denaturation curve from 20 to 94°C was acquired (Figure S3B), and the T_m (melting temperature) was estimated by the Boltzmann model in 66.5°C ± 0.3°C (Figure S3C). Gülich et al. [37] reported the T_m for the Z domain over 75°C, which is 10°C higher than our results, suggesting that the Z6AlkC-P (a 6 concatemer) is less thermally stable than the monomeric version. Thermal denaturation and regeneration were also performed by heating the Z6AlkC-P solution until 94°C and cooling back to 20°C (Figure S3D). The plots showed that after the heating at 94°C, the protein recovered

the initial secondary structure, which indicates a robust folding even after thermal denaturation.

The affinity of Z6AlkC-P toward human IgG was measured using Microscale Thermophoresis (MST) (Figure S4). The determined dissociation constant (K_D) was 39 ± 13 nM, which is within the range reported in the literature for the monomeric version—Z(F30A, N23T) domain in 18 nM [14].

3.2 | Testing the Functionality of Z6AlkC on SulfoLink Coupling Resin

The functionality of the expressed and purified material was tested by immobilizing it on a commercially available chromatography resin (SulfoLink Coupling Resin), which is activated with iodoacetyl groups. We followed the manufacturer's instructions, as the primary aim of this work was to demonstrate the functionality of Z6AlkC rather than to optimize the adsorbent. The purified Z6AlkC-P and Z6AlkC-F were coupled to the iodoacetyl groups of SulfoLink Resin by reacting with the C-terminal cysteine. Similar amounts of protein were immobilized onto the resin, namely Z6AlkC-P (7.2 ± 0.1 nmol/mL packed bed; 0.3 ± 0.01 mg/mL packed bed) and Z6AlkC-F (9.0 ± 0.7 nmol/mL packed bed; 0.4 ± 0.03 mg/mL packed bed) (Figure S5 and Table S5).

Z6AlkC-P and Z6AlkC-F resins bound 6.5 and 3.9 nmol of hIgG/mL packed bed, respectively (corresponds to 1.0 and 0.6 mg hIgG/mL packed bed, Figure S6A, Table S6). For the recovery of hIgG, Z6AlkC-P resin eluted 42% of bound target (2.7 nmol IgG/ mL packed bed; 0.4 mg IgG/ mL packed bed), whereas with the Z6AlkC-F, we recovered 76% of bound hIgG (3 nmol/ mL packed bed; 0.4 mg IgG/ mL packed bed). Z6AlkC-P and Z6AlkC-F resins bound equivalent amounts of pure Trastuzumab (4.2 and 4.1 nmol Trastuzumab/mL packed bed; 0.6 mg IgG/ mL packed bed), and high recovery rates were noted (between 78% and 100%) (Figure S6B and Table S6).

Overall, the performance of the adsorbents containing Z6AlkC-P and Z6AlkC-F was similar, both in terms of the amount of protein immobilized and regarding antibody recovery. The results showed that only 50% of Z6AlkC that were immobilized on the resin (7 to 9 nmol of Z6AlkC/ mL packed bed) bound to the antibody (3.9 to 6.5 nmol antibody/ mL packed bed).

Table 1 demonstrates that the ultradiafiltration process required less time and processed a larger volume (18.5 mL/min) compared to purification using IgG affinity chromatography (0.1 mL/min). Despite the cost of the adsorbent, ultradiafiltration reduces the water consumption by 22×, when compared with affinity chromatography. Considering that ultradiafiltration has higher productivity, we proceeded with the characterization of the Z6AlkC-F adsorbent.

3.3 | Assessing Ligand Density Z6AlkC on SulfoLink Coupling Resin

To achieve the best performance of Z6AlkC-F adsorbent, different Z6AlkC-F concentrations (0.8, 2.7, 6.7, and 14.0 mg of Z6AlkC-F per mL of packed bed, that is, 19, 68, 167, and 351 μM of Z6AlkC-F, respectively) were immobilized on the SulfoLink Coupling Resin, in a small scale (200 μL of packed bed). This variation yielded adsorbents with distinct ligand densities (Table 2).

Then, the four distinct adsorbents were further incubated with pure human IgG to evaluate their performance (Figure 3 and Table 3). These results allowed the selection of the ideal ligand density, which corresponded to the best performance of the adsorbent, that is, resin with a load concentration of 2.7 mg of Z6AlkC-F/mL packed bed. The statistical testing for key comparisons was included in Figure S7.

This best-performing adsorbent (2.7 mg of Z6AlkC-F/mL packed bed load) was further tested at a larger scale (1 mL) using an AKTA chromatography system. A full breakthrough curve was recorded to determine the dynamic binding capacity ($DBC_{10\%}$) of Z6AlkC-F on SulfoLink for Mab1, yielding a value of 9.5 mg/mL (Figure 4A and Table 4). Based on this result, an equivalent amount of Mab1 (Figure 4C) was further loaded in the 1 mL column, resulting in 89% recovery (Table 4). To evaluate the selectivity of the resin, 20 mL of Mab2 was loaded (Figure 4E), leading to an IgG recovery of 83% (Table 4) with high purity (as assessed by SDS-PAGE, Figure S8). Between each run, a cleaning-in-place (CIP) step was performed using 0.1 M NaOH, and no loss of adsorbent activity was observed. These experiments confirmed the findings from gravitational studies, demonstrating that the resin is highly efficient in both binding and eluting IgG while maintaining strong selectivity.

3.4 | Testing the Functionality of Z6AlkC-F on Epoxy Resin

We also immobilized the Z6AlkC-F in an alternative resin (Toyopearl resin), through its available epoxy groups for reaction. The immobilization was performed at pH 6.5 to promote the reaction between epoxy and thiol groups following a previously described protocol [25]. The ligand density was 0.4 mg Z6AlkC per mL of settled bed, and the adsorbent was further characterized. The dynamic binding capacity ($DBC_{10\%}$) of Z6AlkC-F on Toyopearl resin (2.67 mL column) was assessed with a breakthrough curve experiment (Figure 4B), yielding a value of 6.8 mg/mL of settled bed (Table 4).

For the run with purified IgG1 (Trastuzumab), the adsorbent bound 85% (5.8 mg/mL settled bed), and 64% recovery was achieved (3.7 mg/mL settled bed) (Figure 4D and Table 4). For the harvest run (IgG Trastuzumab containing cell culture supernatant from CHO cultivation), the adsorbent bound 58% (4.5 mg/mL settled bed) of IgG, of which 59% were recovered (2.6 mg/mL settled bed) (Figure 4F and Table 4). The stability and reuse after the clean-in-place (CIP) step, which includes treatment with an alkaline solution, showed that the performance of the adsorbent was not affected by the CIP (Figure S9). The IgG binding assays performed with Tris-blocked resin showed no detectable IgG binding, confirming that the blocking procedure effectively eliminated nonspecific interactions.

Taken together, SulfoLink resin exhibited a higher $DBC_{10\%}$ than Toyopearl (9.5 and 6.8 mg/mL settled bed, respectively) and

TABLE 2 | Efficiency of immobilization of Z6AlkC-F to achieve distinct ligand densities.

Load Z6AlkC (mg/mL packed bed)	Load Z6AlkC (nmol/mL packed bed)	Z6AlkC Immobilized (mg/mL packed bed)	Z6AlkC Immobilized (nmol/mL packed bed)	% Immobilized
0.8 ± 0.003	19 ± 0.1	0.2 ± 0.01	4 ± 0.2	23 ± 1
2.7 ± 0.02	68 ± 0.4	1.1 ± 0.06	27 ± 1	40 ± 2
6.7 ± 0.1	167 ± 3	0.9 ± 0.1	23 ± 3	14 ± 1
14.0 ± 0.4	351 ± 9	5.1 ± 0.1	127 ± 3	36 ± 0.04

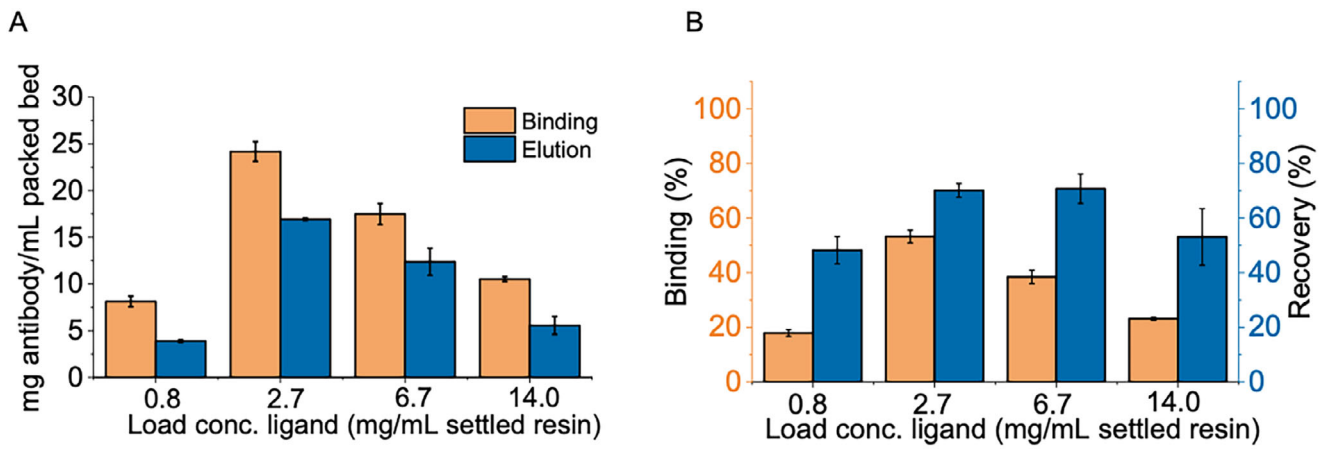

FIGURE 3 | Performance of Z6AlkC-F adsorbents with different ligand densities (Z6AlkC-F) toward binding and elution of pure human IgG. Results of the binding (orange) and elution/recovery (blue) in (A) mg of antibody per mL of packed bed and (B) in percentage (%) with pure human immunoglobulin G (hIgG). The percentage (%) of recovery is based on the bound amount of human IgG. The mean of technical triplicates ($n = 3$) is shown; error bars indicate the standard deviation.

TABLE 3 | Performance of Z6AlkC-F adsorbents with different ligand densities (Z6AlkC-F) to bind and elute pure human IgG.

Load Z6AlkC (mg/mL packed bed)	Load hIgG—target (mg/mL packed bed)	Binding hIgG (mg/mL packed bed)	% Bound	% Recovery
0.8 ± 0.003	45.4 ± 0.6	8.1 ± 0.6	18 ± 1	48 ± 5
2.7 ± 0.02		24.2 ± 1.1	53 ± 2	70 ± 3
6.7 ± 0.1		17.5 ± 1.1	38 ± 2	71 ± 5
14.0 ± 0.4		10.5 ± 0.3	23 ± 1	53 ± 10

Mean ± standard deviation of technical triplicates ($n = 3$) shown.

bound more IgG1 (9.1 and 5.8 mg/mL settled bed, respectively), of which more was subsequently recovered (89% and 64%, respectively).

4 | Conclusions

Significant advancements have been made in the production and purification of *Staphylococcus aureus* protein A (SpA), as an affinity ligand for bioseparation. Although various commercial SpA-like resins are well-documented, the upstream production of alkali-resistant and high-binding capacity SpA variants remains underexplored. The production titers for the hexaconcatemeric alkali-resistant Z domain, especially in high cell density, carbon-limited bioreactor cultures, have not yet been reported. In this work, we efficiently produced a SpA protein variant secreted into the extracellular medium as a soluble protein through a high cell density, carbon-limited bioprocess. We coupled our bioprocess to an ultradiafiltration approach for Z6AlkC purification, contributing to limiting the costs and downtimes to produce IgG purification resins. We first generated the six concatemeric head-to-tail repeats of monomers of the alkali-resistant variant of SpA domain B (Z6AlkC protein). We scaled up the production from shake flask cultures to a 1 L fed-batch bioreactor culture with two identical fermentation cultures. We produced up to 150 mg/g secreted Z6AlkC per cell dry mass (CDM)—or 4 g/L secreted Z6Alk per CDM—and 100 mg/g CDM (3 g/L) soluble Z6AlkC at high cell density (27 g/L). The protein was produced mostly in the culture medium with approximately 60%–70% secreted Z6AlkC, 30%–40% in the soluble fraction, and 0% in the

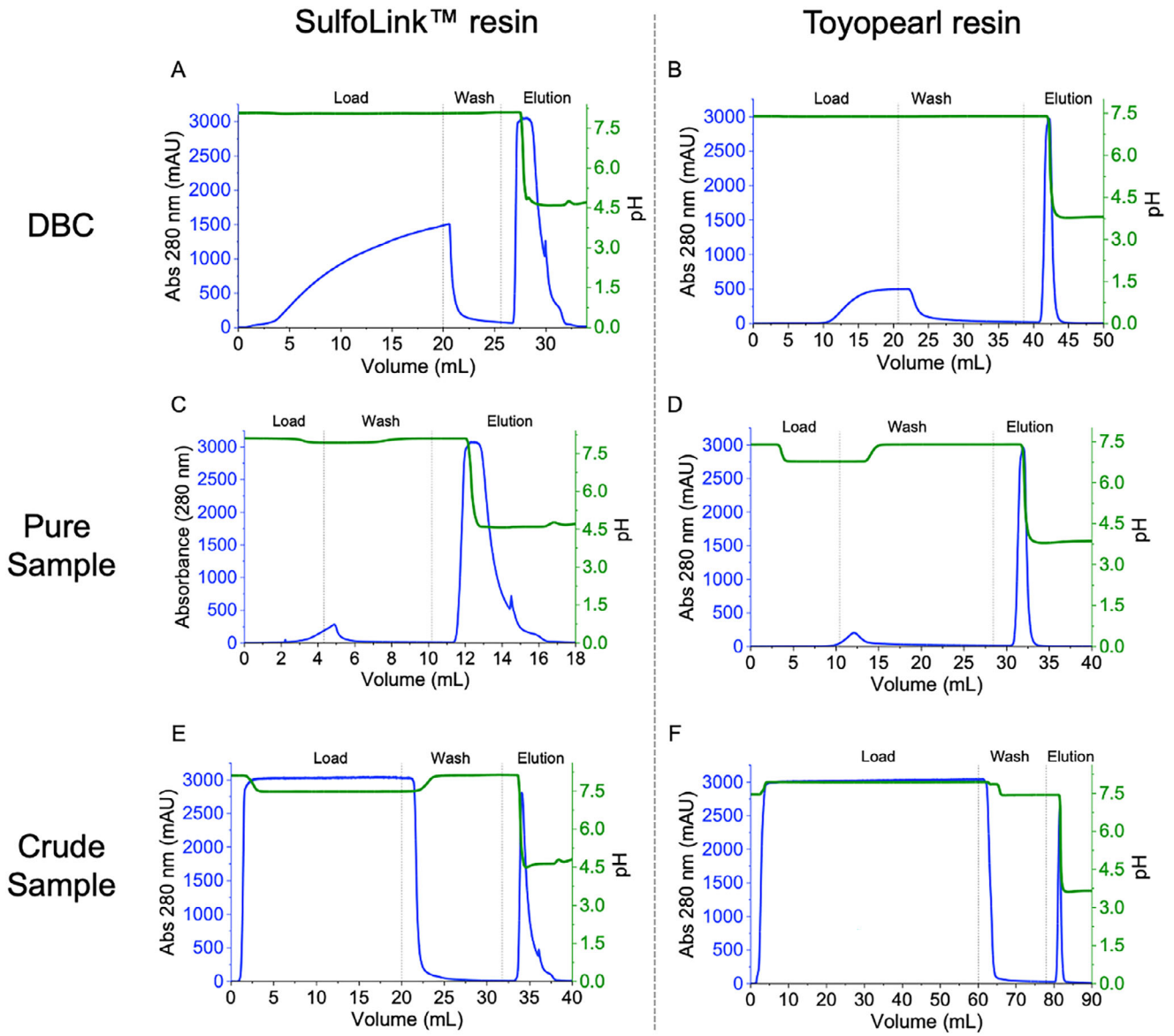

FIGURE 4 | Chromatograms of the Z6AlkC-F immobilized on SulfoLink resin (left) and Toyopearl resin (right). Full breakthrough curve of $DBC_{10\%}$ (A, B) performed with pure IgG1 (Mab1). Run with 10% full breakthrough with pure IgG1 (Mab1) (C, D) and harvest (E, F) IgG1 (Mab2). The UV absorbance at 280 nm is displayed via a solid blue line, and the pH via a solid green line. Chromatographic method steps (load, wash, elution) are indicated on top of the respective chromatogram.

insoluble fraction, both in shake flask and bioreactor cultures. Secreting the recombinant protein simplified purification, avoiding cytoplasmic contamination, and reduced proteolytic activity, which streamlined our recovery process. With a purity of 89% of Z6AlkC in the secreted fraction, we decided to apply and compare two different purification methods: affinity chromatography on IgG Sepharose 6 Fast Flow and ultradiafiltration, resulting in product recoveries of 98% and 99% with a purity of 93% and 89%, respectively. In terms of volumetric throughput, for the given lab-scale set-up, ultradiafiltration was the favorable method, whereas, when aiming for a higher product purity, chromatography should be chosen.

We performed proof-of-function of Z6AlkC-P and Z6AlkC-F as ligands in chromatographic adsorbents. Their performance was similar in terms of the ligand immobilized (7.2 and 9.0 nmol/g agarose beads, respectively) and also in terms of antibody recovery. Considering that the purification of Z6AlkC-F was less time-consuming at a lower cost, and that the performance as an affinity ligand was similar to Z6AlkC-P, we further characterized the Z6AlkC-F adsorbent. We optimized the ligand density in the resin and evaluated the immobilization of Z6AlkC-F on two different commercially available chromatographic adsorbents, demonstrating the activity and suitability of the produced ligand for antibody purification applications.

We consider that ultradiafiltration is an ideal method to obtain high volumetric throughput with a less time-consuming process, while chromatography is preferred when higher product purity is required. However, after conjugation in two commercial chromatographic resins, both affinity ligands (Z6AlkC-F and Z6AlkC-

TABLE 4 | Characterization of Z6AlkC-F immobilized in SulfoLink and Toyopearl resin. Dynamic binding capacity ($DBC_{10\%}$), in mg per mL of settled bed, and performance with pure IgG1 (Mab1) and harvest IgG1 (Mab2).

		SulfoLink Resin	Toyopeal Resin
Ligand density (mg Z6AlkC-F/mL settled bed)		1.1	0.4
$DBC_{10\%}$ (mg/mL settled bed)		9.5	6.8
Pure IgG1	Load (mg/mL settled bed)	9.2	6.8
	Bound (mg/mL settled bed)	9.1	5.8
	Eluted (mg/mL settled bed)	8.1	3.7
	Recovery (%)	89	64
Crude IgG1	Load (mg/mL settled bed)	4.3	7.7
	Bound (mg/mL settled bed)	4.2	4.5
	Eluted (mg/mL settled bed)	3.5	2.6
	Recovery (%)	83	59

P) efficiently captured human IgG and recovered with 70% to 89%, even from crude samples. In addition, the resins were stable after standard clean-in-place procedures and could be reused without loss of performance. Thus, we successfully developed a bioproduction strategy that improves Z6Alk production yields, shortens purification process times, and contributes to streamlining the development of protein A-based chromatography processes.

Author Contributions

C. M. Natal and C. Lacombe contributed equally as first co-authors. They contributed with investigation, formal analysis, writing – original draft, writing – review & editing. A. Zollner, C. Domingos, and J. Mendes contributed with investigation, formal analysis, and writing – original draft. A. Nascimento contributed with investigation, supervision, and writing – original draft, writing – review & editing. A.M.G.C. Dias contributed with supervision, writing – original draft, writing – review & editing and A.J.M. Barbosa contributed with formal analysis. C. Peixoto, B. Wiltschi, A. Jungbauer, A.C. A. Roque contributed with conceptualization, supervision, methodology, writing – review & editing and funding acquisition.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The original contributions presented in the study are included in the Supporting Information; further inquiries can be directed to the corresponding authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: biot70247-sup-0001-SuppMat.docx.