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Technical Note

Protein G affinity chromatography is an underrated but very potent purification method for a broad range of species-independent and tag-less Fab-fragments

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ABSTRACT

Because of their superior properties for certain biological applications small antibody derivatives like fragment of antigen binding (Fab) have found widespread use in basic research and as therapeutics. However, generation of Fab-fragments is still a rather complex matter, reflected by the fact that a variety of methods and purification techniques are necessary for the production of all the different classes of Fab-fragments (kappa/lambda light chains, type of species). Here we demonstrate that Fab-fragments derived from six different antibodies of human or murine origin produced by transient expression in HEK cells can be purified in a single step to a high degree of purity by standard protein G affinity chromatography. This is most likely due to alternative contact sites for protein G located in the CH1 domain of the Fab heavy chain. Our data demonstrate that protein G affinity chromatography as for whole antibodies is a robust method for the purification of tag-less Fab-fragments independent of species, significantly simplifying the process of Fab-fragment purification.

1. Introduction

In basic research, diagnostics, and for therapeutic applications antibodies are indispensable. By genetic engineering it is nowadays possible to manipulate antibodies conveniently in many different ways. For example, antibodies derived from animals can be humanized for therapeutic purposes, antigenic peptides can be added for antigen targeting in vivo, the iso/subtype can be switched, they can be multimerized, or engineered as bispecific or multispecific molecules recognizing two or more different antigenic epitopes simultaneously. Antibodies can be produced in transient or stable mammalian expression systems in huge amounts and in a wide variety of different formats either as whole antibodies or as small antibody derivatives like scFv, nanobodies or Fab-fragments (Brinkmann and Kontermann, 2017).

For certain research applications the use of down-sized antibodies like Fab-fragments is superior to the use of whole antibodies. Due to their smaller size Fab-fragments show improved tissue penetration making them better candidates for immunohistochemistry or the treatment of certain tumors (Beckman et al., 2007). Since Fab-fragments are devoid of the Fc-domain they do not mediate immune effector functions by binding to Fc-receptors or by activation of complement and can replace whole antibodies, wherever immune effector functions are undesirable. Furthermore, because Fab-fragments are monovalent they are best suited for the determination of binding affinities and have proven to be particularly suitable as chaperones for the structure determination of crystallization resistant proteins (Koide, 2009).

Fab-fragments are composed of a light chain (VL + CL, kappa or lambda) linked by a disulfide bond to a shortened heavy chain (VH + CH1), termed the Fd-fragment. The most common method for the generation of Fab-fragments still involves digestion of antibodies with proteolytic enzymes (e.g. papain), separating the Fab-fragment from the Fc-part (Porter, 1959; Brezski and Jordan, 2010). Despite the availability of diverse commercial antibody fragmentation kits generation of sufficient amounts of Fab-fragments by digestion can be very tedious. Because antibodies differ in their sensitivity to proteolytic enzymes, digests are not always complete, resulting in low yield. Therefore, various parameters like amount of enzyme, time of digest, and temperature need to be optimized and after the digest the Fab-fragments have to be separated from the Fc-fragments by an additional purification step.

An alternative method is the genetic engineering and expression of recombinant Fab-fragments in cell-based systems. Due to their small

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molecule size, *E.coli* is the organism of choice as expression host, but it has certain disadvantages. Besides the problem of endotoxin contamination, expression of Fab-fragments in *E.coli* is accompanied by a more or less complicated refolding process, removal of byproducts like misfolded molecules or aggregates, and does not necessarily work for all antibodies (Patil et al., 2022). Expression of Fab-fragments in eukaryotic cells can circumvent these problems leading to correct protein folding and posttranslational modifications as has been described in previous publications (Vazquez-Lombardi et al., 2018).

Whereas the purification of whole IgG antibodies (the most widely used isotype) is a straightforward process, the purification of Fab-fragments can still be improved. Whole IgG antibodies, independent of their species, bind very tightly to staphylococcal protein A or streptococcal protein G via a high affinity binding site located in the Fc-domain (Kato et al., 1995). Therefore, whole IgG antibodies can be purified highly efficiently in a single step by affinity chromatography with easy-to-use protein A or G columns. Since Fab-fragments by definition lack the Fc-domain, purification via protein A or G cannot be considered a generic method for Fab-fragment purification.

Since a common matrix suitable for the purification of all classes of Fab-fragments is not available, various alternative purification matrices and strategies have been developed. For example, Fab-fragments containing variable domains of certain kappa light chain subclasses can be purified by protein L affinity chromatography (Nilson et al., 1992). Columns with affinity ligands specific for subdomains of the light chain (kappa or lambda) or the Fd-fragment (e.g. Capture Select columns from ThermoFisher), are mainly suitable for the purification of human Fab-fragments. Other purification strategies make use of a combination of different purification techniques including ion exchange chromatography and gel filtration. Some of the most common methods are based on the addition of purification tags (e.g. His- or StrepTag) to the C-terminus of the Fd-fragment (Zhao et al., 2009). However, this is unfavorable for downstream experiments, since the influence of tags on the outcome of an experiment is unpredictable, especially when performed in vivo.

In the eighties and nineties of the last century additional contact sites for protein G located in the CH1 domain of IgG antibody heavy chains, conserved across many species and IgG subclasses, have been identified (Erntell et al., 1988; Derrick and Wigley, 1994). These additional contact sites form a low affinity binding surface for protein G that is in the low μM range as compared to the high affinity binding site ($K_D \sim 10$ nM) located within in the Fc-domain (Bailey et al., 2014). However, the almost complete absence of publications in the recent literature on exploiting the CH1-domain for Fab-purification and the plethora of different methods and resins developed for the purification of Fab-fragments indicates that these earlier findings seems to have received little attention.

We speculated that, despite its low affinity, this alternative CH1 binding surface could be used for the routine purification of Fab-fragments via protein G affinity chromatography. To this end we cloned Fab-fragments from six different antibodies of human or murine origin and transiently transfected HEK-cells. All Fab-fragments were produced and could be isolated from the culture supernatants in a highly pure form using protein G columns. Our data suggest, that protein G can be used as a common matrix for the purification of Fab-fragments across different species, making the addition of artificial purification tags and the use of different purification matrices obsolete.

2. Materials and methods

2.1. Antibodies

Human antibodies COVOX-222 and COR-101 (both directed against SARS-CoV2-spike protein) have been described in references Dejnirattisai et al., 2021 and Bertoglio et al., 2021, respectively. The sequences of the light and heavy chain variable regions of COVOX-222 and COR-101 were obtained from PDB database entries 7NX6 and 7B3O, respectively. Mouse antibodies AP406, AP430 (both directed against the plant toxin abrin derived from *Abrus precatorius*, Worbs et al., 2021), SEI92 and SEH449 (both directed against *Staphylococcus aureus* enterotoxins, Dettmann et al., unpublished) were generated in-house by hybridoma technology. Sequences of the variable regions of light and heavy chains were determined from hybridoma with the help of V-region specific primer libraries and RT-PCR.

2.2. Cloning and expression of antibody Fab-fragments

Fab-fragment light chains (kappa) were composed of a signal peptide, VL and CL domain. Fab-fragment heavy chains were composed of a signal peptide, VH domain, CH1 domain of human or mouse IgG1 and part of the upper hinge region. In some constructs a 6× HisTag was added via a GS-linker to the C-terminus of the hinge region (see Fig. 1B). DNA molecules encoding the light and heavy chains of human (COVOX-222, COR-101), mouse-human chimeric (AP406, AP430), and mouse (SEI92, SEH449) Fab-fragments were synthesized by GeneArt (ThermoFisher Scientific, Dreieich, Germany) as human codon-optimized sequences. The light and heavy chains were transferred separately by restriction cloning into the mammalian expression vector pTT5® (under licence from National Research Council of Canada). For recombinant protein expression, pTT5® plasmids (light and heavy chains in a 1:1 ratio) together with polyethyleneimine (Polysciences, Warrington, PA) were used to transiently transfect HEK 293-6E cells (National Research Council of Canada) grown as suspension culture in shake flasks in FreeStyle™ F17 expression medium (ThermoFisher Scientific, Dreieich, Germany) for 6–7 days at 37 °C and 5% CO_2. The secreted Fab-fragments were purified from the culture supernatants by affinity chromatography with 5 mL HiTrap Protein G HP columns using an Äkta avant 25 chromatography system (both Cytiva, Freiburg, Germany) and a flow rate of 3 mL/min. Phosphate buffered saline (PBS) pH 7.3 was used as binding buffer. Elution was performed with 0.1 M glycine buffer pH 2.7. Purified Fab-fragments were buffer-exchanged and stored in PBS. Concentration was determined by measuring A_{280} using a NanoPhotometer (Implen, Munich, Germany). The corresponding recombinant full-length antibodies were produced for comparison in the same expression system and purified by protein A or protein G affinity chromatography. COVOX-222 and COR-101 were expressed as human IgG1, AP406 and AP430 as mouse-human chimeric IgG1 antibodies. SEI92 was expressed as mouse IgG1 and SEH449 as mouse IgG2a antibody.

2.3. Characterization of Fab-fragments by SDS-PAGE and size exclusion chromatography

10 μL cell culture supernatant, 10 μL column flow through, and 10 μg of each purified Fab-fragment were separated on 10 or 12% SDS-polyacrylamide gels either under reducing or additionally under non-reducing conditions. Proteins were stained with the Quick Coomassie stain from Protein Ark (Rotherham, UK). Densitometric analysis of the protein bands in the polyacrylamide gels was carried out with the Image Lab software from BioRad. Analytical size exclusion chromatography was carried out on a Superdex 200 Increase 10/300 GL column (Cytiva, Freiburg, Germany) with 400–500 μg protein in a volume of 250 μl PBS buffer at a flow rate of 0.75 mL/min.

2.4. Surface plasmon resonance (SPR) measurements

Binding kinetics and affinities for the interactions between different recombinant whole IgG antibodies or Fab-fragments and protein G were determined by SPR measurements. For this purpose, we immobilized Pierce recombinant protein G (ThermoFisher Scientific, Dreieich, Germany) on the surface of a Series S Sensor Chip CM5 using an Amine Coupling Kit (both Cytiva, Freiburg, Germany) and standard EDC/NHS coupling chemistry. Immobilization was carried out on flow cell 2 by

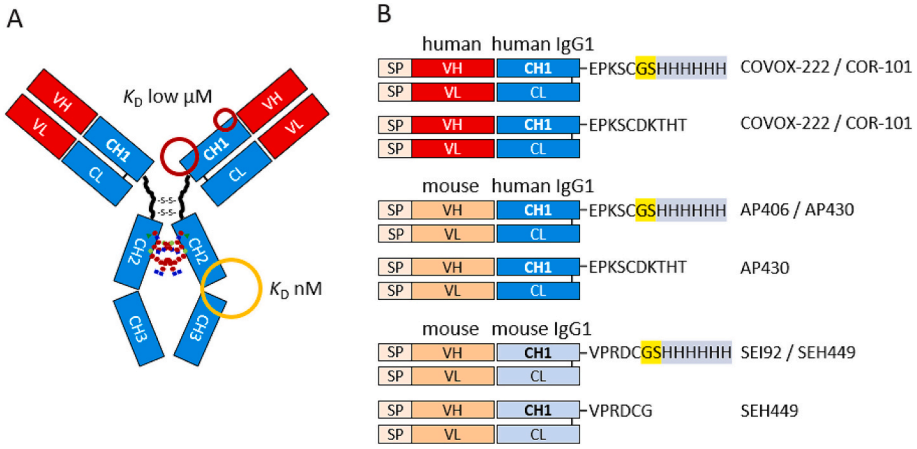

Fig. 1. Streptococcal protein G binding regions of IgG and the various Fab-fragments used in the experiments. A. Scheme of an IgG antibody showing the location of the high affinity binding site (orange circle) and the low affinity contact sites (red circles) for protein G (Derrick and Wigley, 1994). B. Schematic representation of the human (COVOX-222, COR-101), mouse-human chimeric (AP406, AP430), and mouse (SEI92, SEH449) Fab-constructs, with or without 6× HisTag and GS-linker (yellow) used in the experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diluting protein G in 10 mM acetate buffer (pH 4.0) to a concentration of 2 µg/mL using an immobilization time of 7 min at a flow rate of 10 µL/min, which resulted in 59.3 RU of immobilized protein G. Flow cell 1 was activated and deactivated, serving as reference flow cell in a dual-channel setup (flow cell 2 minus flow cell 1). Similar to the recombinant protein G coupled to the HiTrap Protein G HP column used for Fab-fragment purification protein G from Pierce also contains two Fc-binding domains per protein.

Binding kinetics were determined using single-cycle kinetics by injecting increasing concentrations in a 1:3 dilution series of either Fab-fragments or IgG antibodies. The highest concentrations used were 15 µg/mL (300 nM for 50 kDa Fab and 100 nM for 150 kDa IgG), the flow rate was set to 30 µL/min, with association times of 120 s and dissociation times of 600 s. The whole antibodies and Fab-fragments were diluted in HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20, pH 7.4) running buffer, and the measurements were performed at 25 °C using a Biacore T200 apparatus (Cytiva, Freiburg, Germany). Between measurements, the sensor surface was regenerated by injecting 10 mM glycine-HCl (pH 1.5) for 30 s at a flow rate of 30 µL/min. Binding kinetics (association rate constant k_a and dissociation rate constant k_d) were determined by fitting a 1:1 Langmuir binding model to double-referenced binding curves (Myszka, 1999) for the measured Fab-fragments, or a bivalent analyte model for the measured whole IgG antibodies. Equilibrium dissociation constants K_D were calculated as the ratio between k_d and k_a. Only Fab-fragment of antibody AP430 was fitted to a heterogeneous ligand model due to some nonspecific binding. For analysis and plotting, only the predominant low-affinity component of the interaction was taken into consideration. All measurements were performed as technical duplicates.

3. Results

To demonstrate that the low affinity binding surface for protein G located in the CH1 domain of antibody heavy chains (Fig. 1A) can be exploited for the routine purification of Fab-fragments by protein G affinity chromatography we generated various Fab-fragments, which were either completely human (COVOX-222, COR-101), mouse-human chimeric (AP406, AP430), or completely murine (SEI92, SEH449) (Fig. 1B). At the beginning of our project, Fab-fragments were initially intended to be purified via immobilized metal affinity chromatography (IMAC) and were therefore equipped with a HisTag. Later on, Fab-fragments were constructed without HisTag for comparison and to exclude any influence of the tag on the outcome of the experiments (Fig. 1B). All Fab-fragments could be expressed in HEK cells with a production rate ranging from 16 to 67 mg protein per liter culture supernatant (Table 1). This is less as compared to the yield obtained for the corresponding full recombinant antibodies (Table 1). However, the molecular weight of Fab-fragments is only a third of full IgG antibodies suggesting that HEK cells can produce Fab-fragments just as efficiently as full IgG antibodies on a molar basis. Without exception all Fab-fragments - independent whether they were human, chimeric or mouse derived - bound to HiTrap protein G columns. SDS-PAGE and comparative densitometric evaluation of the Fab-protein bands in the supernatants and the flow through of the protein G columns showed that 70–95% of the Fab-fragments could be isolated from the cell culture supernatants by a single purification cycle (Fig. 2A). Since the protein G binding surface in the CH1 domain was expected to be of low affinity, we initially tried to elute one of our Fab-fragments (SEH449) from the HiTrap protein G column at milder conditions using 0.1 M sodium citrate buffer pH 3.6. Surprisingly, with this buffer we were not able to elute the Fab-fragment from the protein G column and therefore we used 0.1 M glycine buffer pH 2.7, which is routinely used for elution of whole IgG antibodies, as standard elution buffer in all further experiments. In Fig. 2B the analyses of the protein G purified Fab-fragments by SDS-PAGE under reducing and non-reducing conditions is shown, demonstrating the high purity of the isolated Fab-fragments. When analyzed by size exclusion chromatography our affinity purified Fab-fragments eluted as single major peaks that accounted for 93–97% of the protein applied to the column, indicating that our Fab-fragments are largely free of aggregates (Fig. S1).

Searching for an explanation for the remarkable performance of protein G columns in purifying Fab-fragments, we reexamined the binding kinetics and affinities between protein G and whole IgG

Table 1
Comparison of the yields after 1-step-affinity purification.

	VL-VH / CL-CH1	Fab [mg/L]*	IgG [mg/L]
COVOX-222 - His	human / human IgG1	32.1	130.1
COVOX-222	human / human IgG1	26.3	
COR-101 - His	human / human IgG1	66.9	101.7
COR-101	human / human IgG1	62.6	
AP406 - His	mouse / human IgG1	21.5	28.8
AP430 - His	mouse / human IgG1	65.5	67.2
AP430	mouse / human IgG1	52.8	
SEI92 - His	mouse / mouse IgG1	41.7	117.8
SEH449 - His	mouse / mouse IgG1	19.8	41.5
SEH449	mouse / mouse IgG1	16.3	

* Note: Since the recovery rates after a single purification cycle are between 70 and 95%, we somewhat underestimate the amount of Fab-fragment produced.

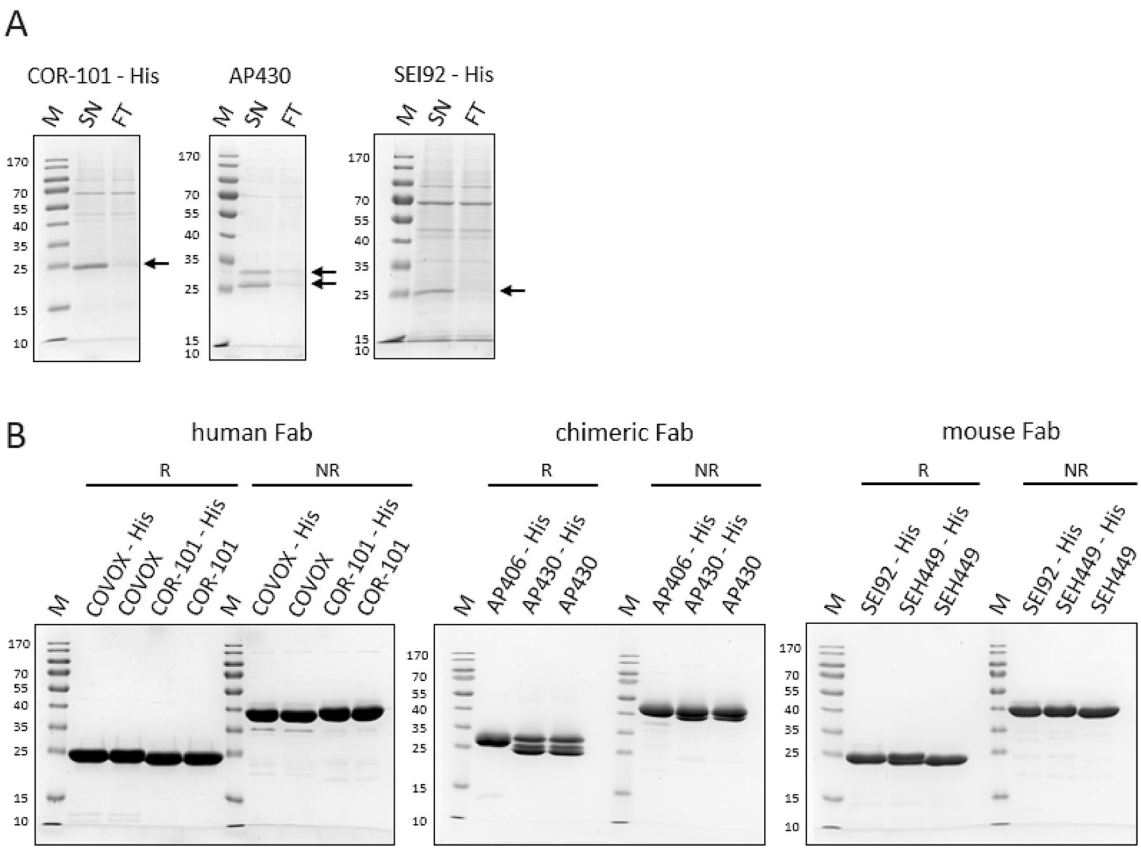

Fig. 2. Biochemical characterization of diverse Fab-fragments isolated from HEK cell culture supernatants by SDS-PAGE.
A. Cell culture supernatants (SN) and column flow throughs (FT; 10 µL each) were analyzed by SDS-PAGE under reducing condition on 10 or 12% polyacrylamide gels. Fab-fragments COR-101, AP430, and SEI92 are shown as representative examples (positions of the Fab-fragments are indicated by black arrows). B. Protein G purified Fab-fragments of human antibodies COVOX-222, COR-101, chimeric antibodies AP406, AP430, and mouse antibodies SEI92, SEH449 were analyzed by SDS-PAGE under reducing (R) or non-reducing (NR) conditions on 12% polyacrylamide gels. In each lane 10 µg protein were loaded. M = PageRuler™ Prestained Protein Ladder (ThermoFisher Scientific).

antibodies or their corresponding Fab-fragments by surface plasmon resonance (SPR) spectroscopy (Fig. 3 and Table S1–3). For whole IgG antibodies we measured K_D values ranging from 0.6 to 0.9 nM for human (COVOX-222, COR-101) and mouse-human chimeric (AP406, and AP430) IgG1, 1.1 µM for mouse IgG1 (SEI92), and 190 nM for mouse IgG2a (SEH449). Interestingly, recombinant Fab-fragments showed K_D values spanning from 330 nM to 3.8 µM. These binding affinities are within the range of affinities observed for whole mouse IgG antibodies, which are routinely purified by protein G affinity chromatography providing an explanation why protein G columns are particularly suitable for purifying Fab-fragments.

4. Discussion

The strategies currently available for the production of Fab-fragments are manifold, complex and often require special expertise for their implementation. Considering their importance for scientific research, a simple and uniform procedure for the production and purification of the various types of Fab-fragments would be of great value. More than 30 years ago an alternative binding surface for protein G in the CH1 domain of IgG antibody heavy chains has been identified in addition to the high affinity binding site located in the Fc-region of IgG antibodies (Erntell et al., 1988; Derrick and Wigley, 1994). Surprisingly, this finding has received little attention for the purification of Fab-fragments until now. Here we demonstrate with Fab-fragments (both human and murine origin) produced by standard transient transfection in HEK cells that the interaction of this binding surface with protein G is sufficiently strong for the reliable purification via protein G affinity chromatography. This finding can be explained by looking at the binding affinities between protein G and Fab-fragments. In accordance with the literature (Bailey et al., 2014) the K_D values for the Fab-fragments analyzed in this work are in the µM range spanning from 330 nM to 3.8 µM. This affinity range is by a factor of approximately 1000 (µM versus nM) lower as compared to human IgG antibodies binding to protein G. However, binding affinities in the µM range are sufficiently strong for affinity purification of proteins. A good example is the Strep-tag technology. The Strep-tag II peptide is a commonly used affinity tag for the purification of recombinant fusion proteins and binds to its immobilized interaction partner Strep-Tactin with a K_D value of approximately 1 µM (Skerra and Schmidt, 1999). More importantly, the binding affinities observed for Fab-fragments and protein G are within the range of whole mouse IgG antibodies (190 nM to 1.1 µM), for which protein G affinity chromatography is the method of choice for purification. The strategy presented here has several advantages compared to alternative production and purification approaches. 1) Fab-fragments can be produced in sufficient quantity in HEK cells within 6–7 days (16–67 mg/L). 2) The often laborious proteolytic digest can be forgone and Fab-fragments produced in HEK cells are already present in their native form in contrast to the expression in *E. coli*. 3) Protein G affinity columns are commercially available, standardized, well known, widely used for the purification of full IgG antibodies, and can be used without any optimization for the purification of Fab-fragments. 4) Fab-fragments can be purified without the addition of potentially interfering artificial purification tags, which often have to be cleaved off, requiring an additional purification step. 5) Besides for human and mouse Fab-fragments protein G affinity chromatography can be most likely used

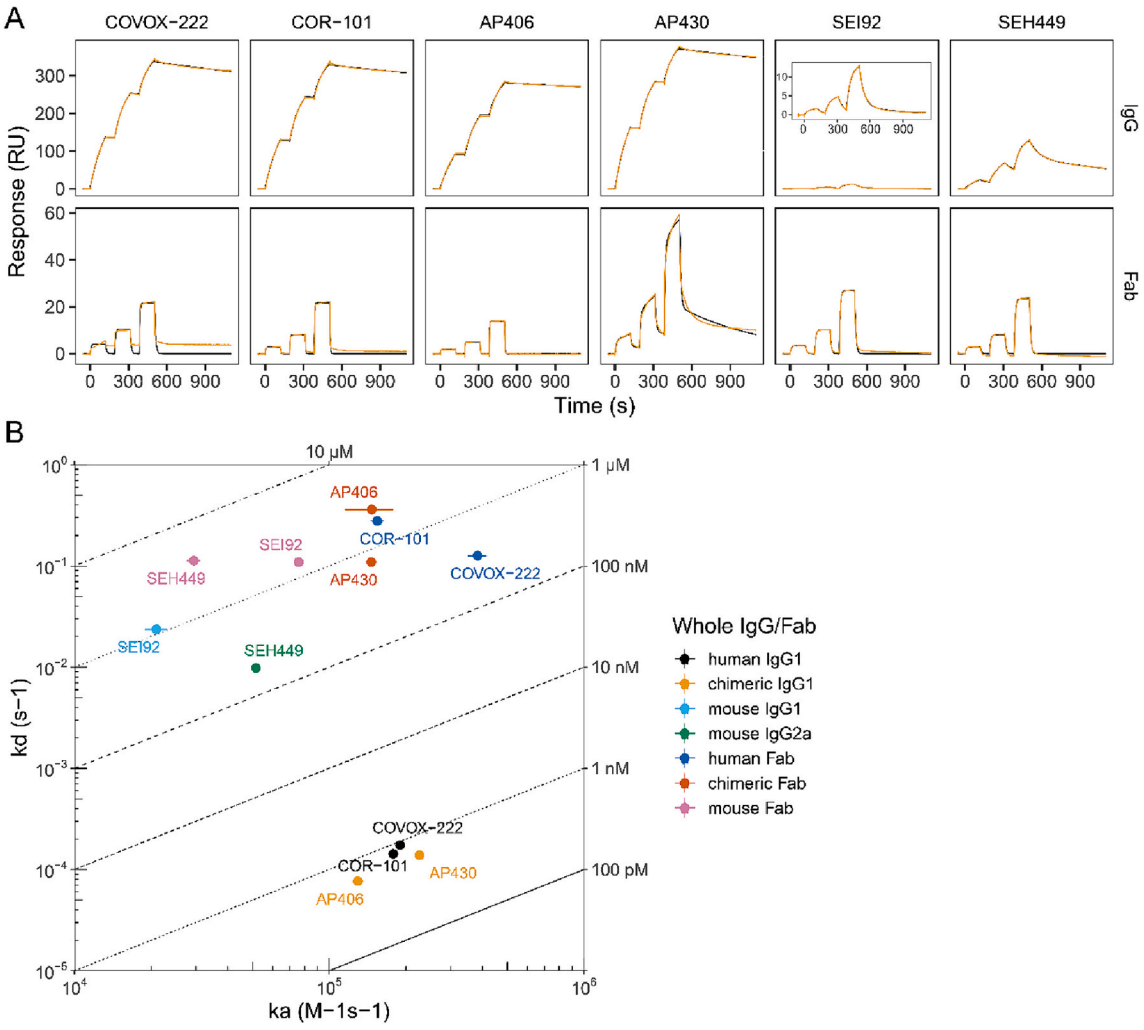

Fig. 3. Binding kinetics and affinities between protein G and whole IgG antibodies or their corresponding Fab-fragments determined by SPR spectroscopy.
A. SPR sensorgrams for the interaction of protein G with whole IgG antibodies and Fab-fragments. Three increasing concentrations (1.67, 5, and 15 µg/mL) of either IgG antibodies or Fab-fragments were injected for 120 s each, followed by a final injection of buffer for 600 s. Double-referenced binding responses (resonance units, RU) are shown for the measured data (beige lines), overlaid with the fitted data from the bivalent analyte model (IgG) or 1:1 Langmuir binding model (Fab). Only Fab-fragment for AP430 was fitted to a heterogeneous binding model (one of two technical replicates is shown). B. Two dimensional isoaffinity plot of rate constants for the tested recombinant whole IgG antibodies and Fab-fragments. Association rate constants k_a (M^{-1} s^{-1}) and dissociation rate constants k_d (s^{-1}) for human IgG1 (COVOX-222, COR-101), mouse-human chimeric IgG1 (AP406, AP430), mouse IgG1 (SEI92), and mouse IgG2a (SEH449) antibodies are shown in comparison to their Fab counterparts (SEH449 Fab, like all other Fab-fragments, was constructed with a IgG1 CH1 domain). The equilibrium dissociation constants K_D are plotted as diagonals (mean value and standard deviation of two technical replicates are shown).

for other species as well, since the binding sites within the CH1 domain are highly conserved across species and IgG subclasses (Derrick and Wigley, 1994). 6) Since the alternative binding surface for protein G is located within the CH1 domain it can be reasonably assumed that purification can be performed independent of the isotype of the light chain (kappa/lambda). 7) Different to matrices that bind Fab-fragments via the light chain (e.g. protein L) protein G affinity chromatography ensures that only correctly composed Fab-fragments are purified, without co-purification of overexpressed light chains. 8) This simple purification approach might well be adapted to other antibody derivatives and fusion proteins containing a CH1-domain.

5. Conclusion

Our data suggest that protein G is a highly underrated universal matrix for the purification of a broad spectrum of different Fab-fragments, superior to other purification matrices, which bind only certain types of Fab-fragments. Fab-fragment expression in HEK-cells and purification by protein G, as described here, is simple, fast, based on standard techniques, and is easily accessible for all laboratories with basic knowledge in protein expression.

CRediT authorship contribution statement

Daniel Stern: Writing – review & editing, Investigation. **Paulin Dettmann:** Resources. **Brigitte G. Dorner:** Supervision, Project administration. **Hans Werner Mages:** Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2024.113669.

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