Using different proteolytic enzymes to digest antibody and its impact on stability of antibody mimetics

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Abstract

There are opportunities to formulate antibodies as solid-state depots for local therapy, which would minimise large systemic doses that are typically required. We have developed antibody mimetics known as Fab-PEG-Fab (FpF) that display similar binding affinity and functional activity as IgG antibodies. For head-to-head comparison between FpF and IgG, FpF is prepared from the Fabs obtained by enzymatic digestion of IgGs. Here, we report for the first time that using different enzymes to proteolytically digest IgG plays an important role in stability profile of the obtained Fabs leading in different stability profiles of the final conjugated product such as FpF. We prepared an anti-vascular endothelial growth factor (VEGF) FpF from either clinical Fab_{rani} (ranibizumab) or Fabs obtained by enzymatic digestion of bevacizumab (IgG) using immobilised papain and gingisKHANTM (KGP) enzyme. The stability of FpFs was then studied after being lyophilised in comparison with both ranibizumab and bevacizumab. Lyophilisation is being evaluated to produce solid material that can be used for depot fabrication. We observed that using immobilised papain to digest IgG resulted in the heterogenous isomers Fab leading to the preparation of heterogenous $FpF_{beva\text{-}papain}$ mimetic that underwent aggregation during lyophilisation. However, using KGP enzyme generated a homogenous intact $Fab_{beva\text{-}KGP}$ as determined by mass spectral analysis. Interestingly, the FpF mimetics prepared from the homogenous Fabs (Fab_{rani} and $Fab_{beva\text{-}KGP}$), displayed greater stability compared to their starting bevacizumab and ranibizumab after being lyophilised as determined by DLS analysis. There is a potential to lypholise FpFs to be used to fabricate solid-state depots.

Introduction

We have developed an antibody mimetic called Fab-PEG-Fab (FpF) (Fig. 1a) [1], which displays similar *in-vitro* and *in-vivo* activities as the corresponding IgG that binds to the same epitope [2]. The two Fabs in an IgG antibody are linked together through the flexible hinge region to (i) facilitate Fab binding to two epitopes spanning a wide spatial range [3] and (ii) allow cooperative binding of the two Fabs. However, the hinge region in IgG is vulnerable to degradation [4] and disulfide scrambling [5]. In antibody mimetic, FpF, the hinge region is replaced with poly(ethylene glycol) (PEG) scaffold. The resulting FpF does not possess a Fc region and also avoids Fc effector derived side effects [8, 9], which are particularly important

when considering treatments for acute inflammatory conditions. The Fab interchain disulfides in the FpF mimetics, are also stabilised by reannealing disulfide bridging conjugation. Disulfide reannealing conjugation and the presence of PEG reduces the propensity of the FpFs to aggregate. Binding affinity properties are enhanced because the flexibility of the PEG slows dissociation of the FpF from its antigenic site resulting in the potential to increase duration of action within the target tissue.

We have recently prepared a solid-state bevacizumab tablet for intraocular implantation [6]. Bevacizumab is an humanized IgG that binds to vascular endothelial growth factor (VEGF) and used in oncology and age macular degeneration (AMD) [7]. Bevacizumab has shown the potential to control scarring when administered into the subconjunctiva following glaucoma filtration surgery (GFS) but clears quickly after subconjunctival injection. There is a need for a slow releasing form of an anti-VEGF antibody solid-state depot that can be placed in the subconjunctiva space of the eye after surgery and maintained its stability during long period of time. We hence wished to study the stability of anti-VEGF FpF mimetic after lyophilisation. if it remains stable, it might then be possible to formulate high enough amount of mimetic as a solid-state tablet to display prolonged duration of action. Lyophilisation (freeze-drying) is one of the well-known method to formulate proteins as a solid-state depot.

Figure (1)

The Fab used to prepare an FpF can be derived directly by the enzymatic digestion of bevacizumab (Fig. 1b) or use a clinical Fab_{rani} (ranibizumab, an anti-VEGF Fab). We first prepared the FpF_{rani} from Fab_{rani} [1] and studied its stability by dynamic light scattering (DLS) and size-exclusion chromatography (SEC) after being lyophilised. While lyophilised FpF_{rani} maintained its stability, the lyophilised Fab_{rani} in its pharmaceutical formulation aggregated/degradated. We then wanted to expand our study to determine stability of lyophilised FpF prepared from bevacizumab. To prepare intact Fab_{beva} with one interchain disulfide bond, we used two enzymes; papain and gingisKHAN^{TM}. Both enzymes efficiently produced Fabs without impacting the integrity of the binding site [10]. Papain is the most common proteolytic enzyme that is used widely, but this enzyme cleaves the IgG hinge region at various sites resulting in a heterogenous Fabs possessing varying residual hinge derived polypeptide still bond to the Fab. We and others [10] have shown the heterogenicity of a papain digested Fab by mass spectroscopy. However, there has not been any study to investigate stability of the Fab obtained by papain digestion and its impact on stability of the conjugated products. There are wide range of applications where Fabs are used for protein conjugations such as antibody drug conjugates, bispecific antibodies and radiolabel antibody fragments [11, 12].

In contrast, a relatively new enzyme called gingisKHAN (KGP) undergoes selective IgG hinge cleavage at a single digestion site resulting in an homogenous intact Fabs (Fab_{KGP}). We aimed to determine if FpFs prepared from Fabs obtained from papain and KGP ($FpF_{beva\text{-}papain}$ or $FpF_{beva\text{-}KGP}$) digestion of the IgG, displayed a different stability profile and whether they were more stable than the IgG during freeze-drying. We have learned that while Fabs obtained from bevacizumab digested by papain and KGP resulted in a single 50 kDa band in SDS-PAGE analysis, but they appeared to have different LC-MS peaks. In this study, we have noted that using homogenous, intact $Fab_{beva\text{-}KGP}$ to prepare FpF conjugates, stability of the FpF maintained during lyophilisation in contrast to bevacizumab which underwent

aggregation and light and heavy chain dissociation after lyophilisation. Hence there is a potential to formulate high amount of FpF as a solid-state depot for ocular application.

Materials and Methods

Bevacizumab (Avastin, 25 mg/mL, Genentech) was purchased commercially. Immobilized 50% papain slurry (4.0 mL) was purchased from Thermo Fisher. GingisKHAN^{TM} (KGP) (10000 unit, for 10 mg IgG) was purchased from Genovis.

Bevacizumab (10 mg, 1.0 mL) was incubated with immbilised papain (400 uL, at 37^{0}C pH 7.4 for 7 h) or with gingisKHAN^{TM} enzyme (10000 unit, at 37^{0}C pH 8 for 1 h) to obtain Fab. Fabs were purified from undigested IgGs and Fc fragments using a protein A column and then characterised using SDS-PAGE analysis, and LC-MS. Anti-VEGF FpFs were then prepared from Fab_{rani}, $FpF_{beva\text{-}papain}$ or $FpF_{beva\text{-}KGP}$ (Figure 1) following the method described in [1]. Ranibizumab, bevacizumab, FpF_{rani}, $FpF_{beva\text{-}papain}$ and $FpF_{beva\text{-}KGP}$ were then subjected to freeze-drying. Primary drying was performed at -20 ^{0}C for 12 hours at 100 µBar, followed by secondary drying at 20 ^{0}C for 2 hours. Aggregation/stability of both FpF_{beva} and bevacizumab was determined using a dynamic light scattering (DLS) DynoPro plate reader II and SEC. Surface Plasmon Resonance (SPR) technique was used to study the binding affinity of antibody mimetics against VEGF. The CM5 chip was functionalised with VEGF (high RU 1000) to study the binding activity of Fabs and FpFs.

Results and Discussion

FpF_{rani} was prepared by bis-alkylation conjugation of anti-VEGF Fab_{rani} (1.0 eq, 1.0 mg/mL) with PEG di(bis-sulfone) reagent **1** (1.0 eq, 10 kDa) [1]. FpF_{rani} was then purified and subjected to freeze-drying in PBS only buffer. Purified FpF_{rani} (before and after lyophilisation) displayed a single band at about 120 kDa molecular weight in SDS-PAGE analysis (Fig. 2a, lanes 1 and 2) indicating no protein degradation occurred during lyophilisation. Dynamic light scattering (DLS) analysis indicated that FpF_{rani} maintained its stability after lyophilisation (Fig. 2b) while lyophilised Fab_{rani} aggregated (Fig. 2c). In addition, there was no HMW peak in the SEC of FpF_{rani} (Fig. 2d), whereas lyophilised Fab_{rani} displayed a high molecular weight (HMW) peak at 11.05 min consistent with aggregation having occurred.

(**Figure 2)**

It was important to study the binding of lyophilised FpF mimetic and whether its antigen binding activity is preserved. It appeared that the FpF_{rani} maintained its binding activity to the VEGF after being lyophilized studied by SPR analysis (Fig. 3).

(**Figure 3)**

To better conduct head to head comparisons of the FpFs with IgGs, it was required to obtain Fab of the IgG by enzymatic digestion. We first used immobilised papain which is the most common enzyme for antibody digestion [1]. Using bevacizumab, $Fab_{beva\text{-}papain}$ was isolated which was used to prepare the corresponding $FpF_{beva\text{-}papain}$ with PEG di(bis-sulfone) reagent **1.** The purified $FpF_{beva\text{-}papain}$ in PBS only buffer was then subjected to freeze-drying to produce a lyophilised cake. Surprisingly, the $FpF_{beva\text{-}papain}$ was aggregated after lyophilisation and multiple peaks were observed by DLS analysis (Fig. 4a). In addition, HMW and degradation peaks were observed by SEC for lyophilised $FpF_{beva\text{-}papain}$ (Fig. 4b). As expected, bevacizumab

in its pharmaceutical formulation displayed multiple DLS peaks indicating aggregation had occurred during lyophilisation (Fig. 4c).

(**Figure 4)**

We considered that a more selective digestion enzyme was required. Papain is a non-specific protease which cleaves IgG above the hinge region at the N-terminal but below the site of the disulfide bond between the light and the heavy chain. Because the hinge region is more exposed and flexible, it is more susceptible for the proteases cleavage. IgG digestion with the endoproteinase papain to obtain Fabs generally suffer from the presence of undigested IgG, over-digestion and lack of homogeneity and reproducibility. In addition, use of unspecific proteases required time-consuming optimizations and further purification of the Fabs to prevent their degradation. In addition, the integrity of interchain and intrachain disulfide bonds presence in Fab region, could get influenced by papain digestion. The cysteine protease gingipain K [13] of the pathogenic anaerobe bacteria *Porphyromonas gingivalis* has recently become commercially available as the recombinant protease GingisKHAN^{TM} (KGP) from Genovis AB (Lund, Sweden). It has been reported gingipain k could hydrolyze human IgG1 at a single site above the hinge region (KSCDK/THTCPPCP) generating a homogenous pool of intact Fabs and crystallizable fragments (Fcs). It is suggested that IgG digestion with GingisKHAN^{TM} is highly specific and quantitative, does not require much optimization. There might be a difference

KGP enzyme was then used to digest bevacizumab to give $Fab_{beva\text{-}KGP}$. The purified $Fab_{beva\text{-}KGP}$ appeared at 50 kDa molecular weight band (Fig. 5, lane 1) in SDS-PAGE analysis similar to $Fab_{beva\text{-}papain}$ (Fig. 5c, lane 2). We learned that while both $Fab_{beva\text{-}papain}$ and $Fab_{beva\text{-}KGP}$ appeared as a single band in SDS-PAGE analysis, LC-MS analysis detected 3 different isomers for $Fab_{beva\text{-}papain}$ (Fig. 5a) and an additional peak at 23446.76 Da for a free light chain as a result of Fab-disulfide reduction. In contrast, $Fab_{beva\text{-}KGP}$ displayed only a single LC-MS peak at 48381.72 Da (Fig. 5b) indicating a homogenous intact Fab.

(Figures 5)

FpF_{beva} was then prepared from $Fab_{beva\text{-}papain}$ and $Fab_{beva\text{-}KGP}$. Using SDS-PAGE gel (Fig. 6, a) for the final purified $FpF_{beva\text{-}papain}$ and $FpF_{beva\text{-}KGP}$, no difference between the two FpFs could be discerned by SDS-PAGE in terms of the band shapes and purities. However, when the $FpF_{beva\text{-}KGP}$ was subjected to freeze-drying in PBS only buffer, interestingly, the $FpF_{beva\text{-}KGP}$ did not aggregate or display any light/heavy chain dissociation and appeared as a single DLS peak was observed (Fig. 6, b).

(Figure 6)

KGP enzyme digested IgG1 between K223 and T224 to produce intact and homogenous Fab in less than 2 hour, leading to the preparation of homogenous antibody mimetic FpF. Using papain to digest IgG could result in a combination of under- and over-digestion which required specific optimisation and chromatographic purification to obtain desire Fabs.

In summary, our results suggest that FpFs are more stable than bevacizumab during lyophilization while maintaining the binding activity to the VEGF. We also learned that sourcing a pure, homogenous and intact Fab to prepare FpF could play an important rule in the stability of final FpF. Since FpF remains stable during lyophilization, there is a potential to lyophilise FpF with high loading in a solid formulation. Highly loaded implants are desired as a means to further increase the duration of action of the implanted drug.

Conflicts of interest

The authors declare no competing financial interest.

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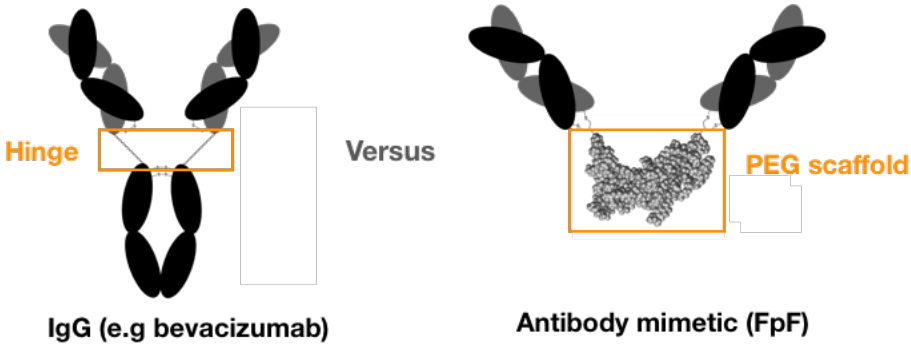
(a) Antibody mimetic (FpF) , the hinge is replaced with PEG scaffold



(b) Enzymatic digestion of IgG to obtain Fabs (either papain or gingisKHAN enzymes) to synthesis FpFs

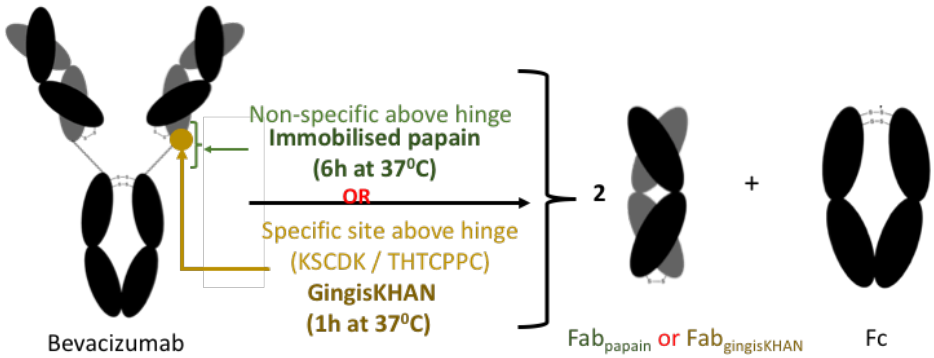


Figure 1. (a) Representation of the Fab-PEG-Fab (FpF) antibody mimetic and an IgG antibody, (b) Enzymatic digestion of IgG using either papain or ginigisKHAN™ which is then followed by purification with protein A to isolate the Fab.

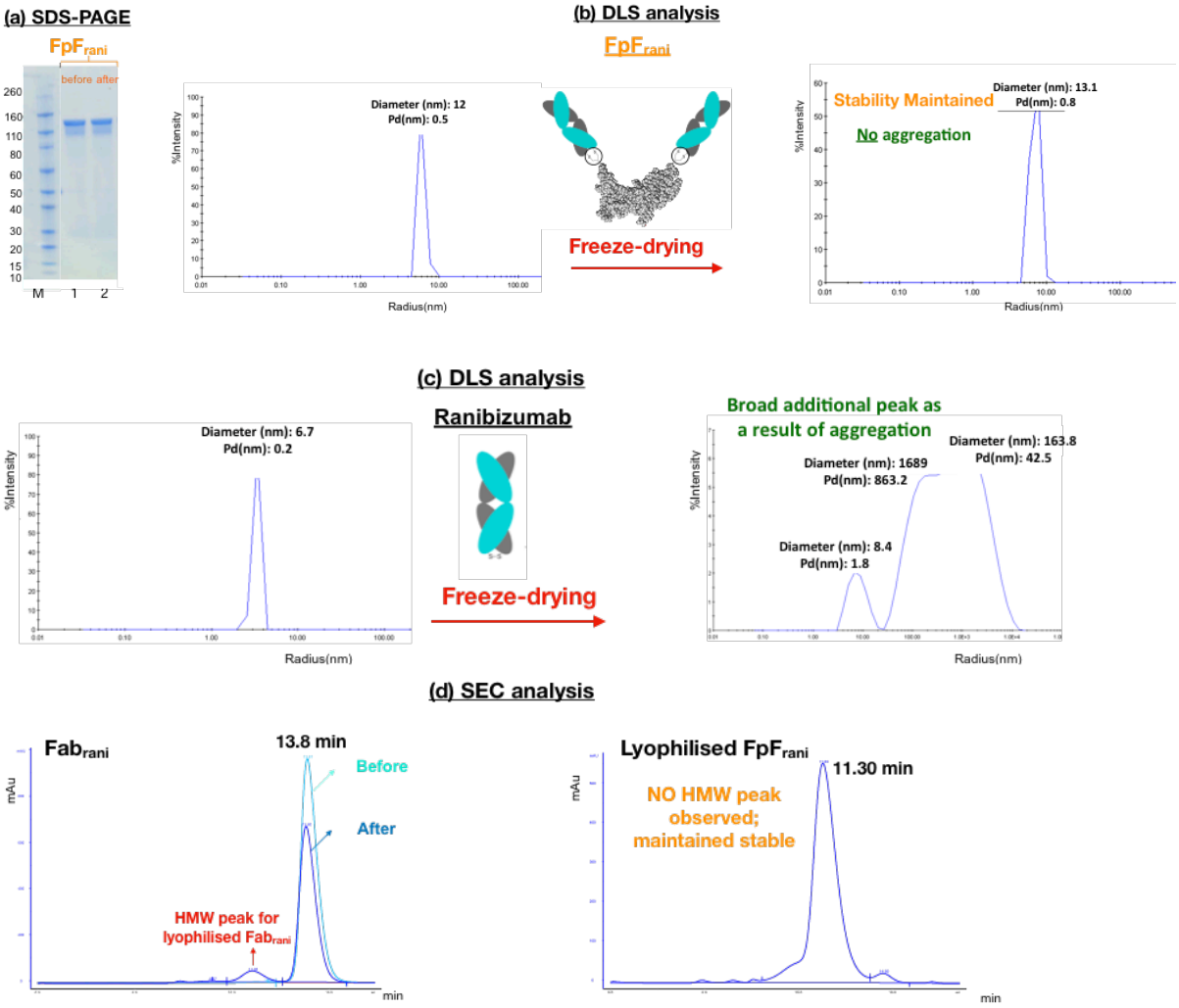


Figure 2. (a) Representative SDS-PAGE for purified FpF_{rani} before and after lyophilisation, (b, c) Representative DLS analysis for Fab_{rani} and FpF_{rani} before and after lyophilisation. While the FpF_{rani} was lyophilised in PBS buffer, the Fab_{rani} was freeze-dried in its pharmaceutical formulation, (d) SEC overlays for Fab_{rani} and FpF_{rani} before and after lyophilisation, which indicate aggregation having occurred for Fab_{rani}. The Fab_{rani} SECs indicate that loss of monomer peak at 13.8 min and presence of HMW peak at 11.05 min. Lyophilised FpF_{rani} maintained its stability studied by DLS and SEC.

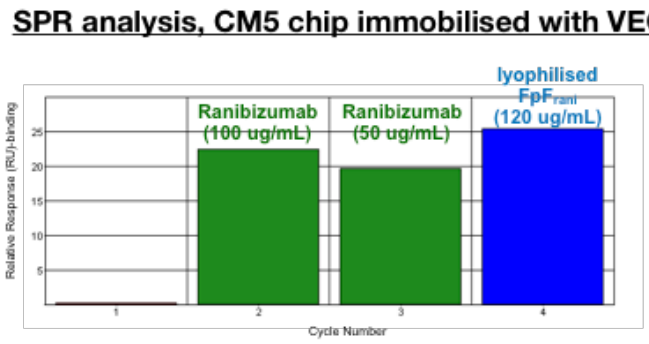


Figure 3. Biacore SPR analysis for Fab_{rani} and lyophilised FpF_{rani}. CM5 chip was immobilised with h-VEGF for binding assay. Lyophilised FpF_{rani} maintained its binding activity to VEGF.

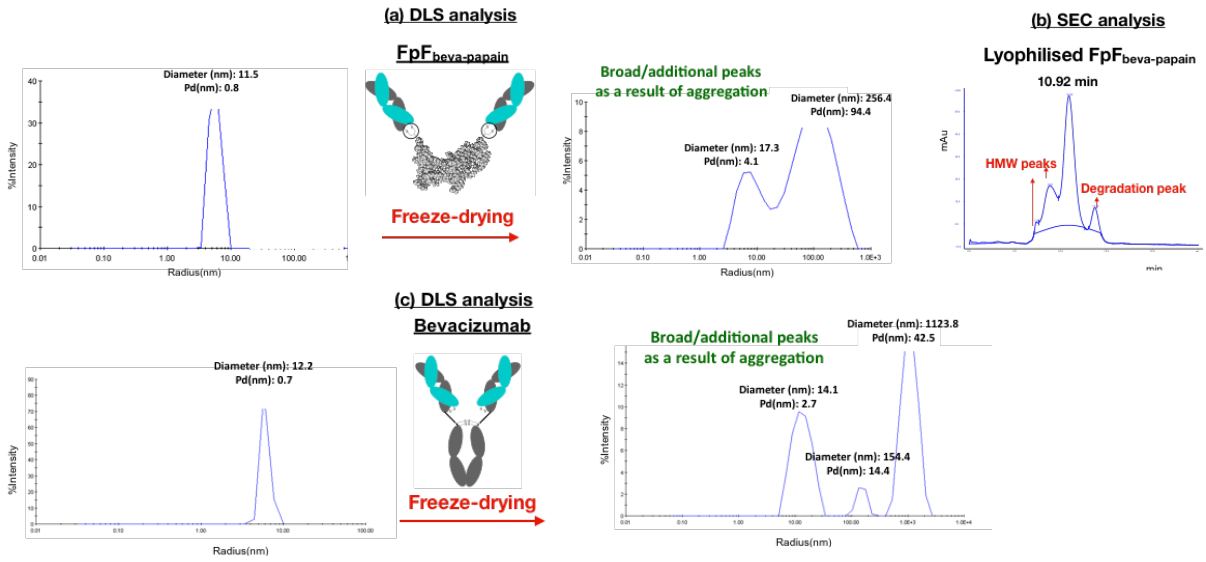


Figure 4. (a, b) Representative DLS and SEC analysis for $FpF_{beva\text{-}papain}$ and **(c)** Representative DLS analysis for bevacizumab before and after lyophilisation. Surprisingly, $FpF_{beva\text{-}papain}$ in PBS buffer displayed aggregation after being lyophilised as multiple peak observed in DLS. SEC also revealed the HMW peaks at 7.02 and 8.3 min and also degradation peak at 11.13 min for lyophilised $FpF_{beva\text{-}papain}$. Bevacizumab, also displayed aggregation when lyophilised being in the pharmaceutical formulation.

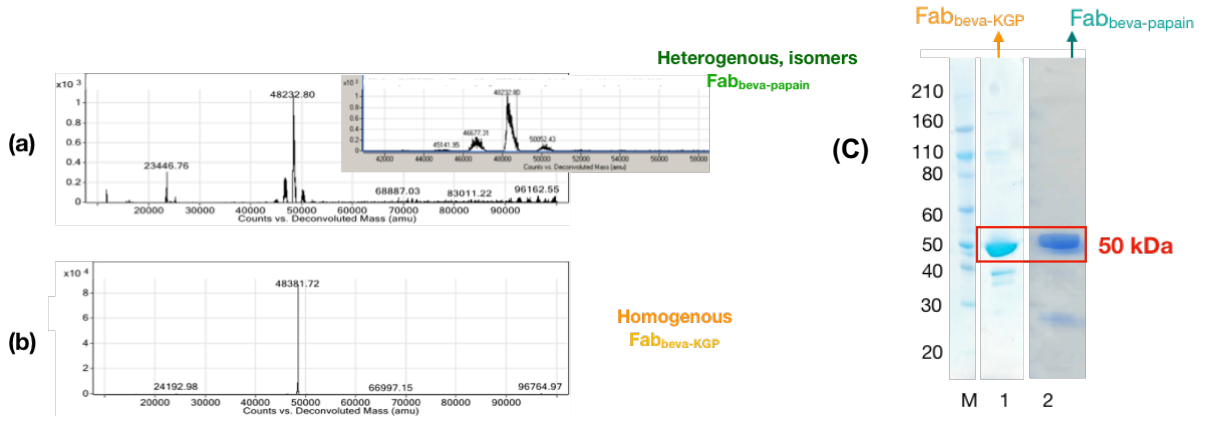


Figure 5. (a, b) LC-MS analysis for purified (a) $Fab_{beva\text{-}apapain}$ and (b) $Fab_{beva\text{-}KGP}$. While $Fab_{beva\text{-}papain}$ resulted in heterogenous three mass spectroscopy peaks, $Fab_{beva\text{-}KGP}$ showed only one LC-MS peak suggesting an homogenous intact pool of Fabs. **(c)** Representative SDS-PAGE gel for purified Fabs obtained from gingisKHAN (lane 1) and papain (lane 2) digestion of bevacizumab. A single band at 50 kDa appeared for both $Fab_{beva\text{-}KGP}$ and $Fab_{beva\text{-}papain}$.

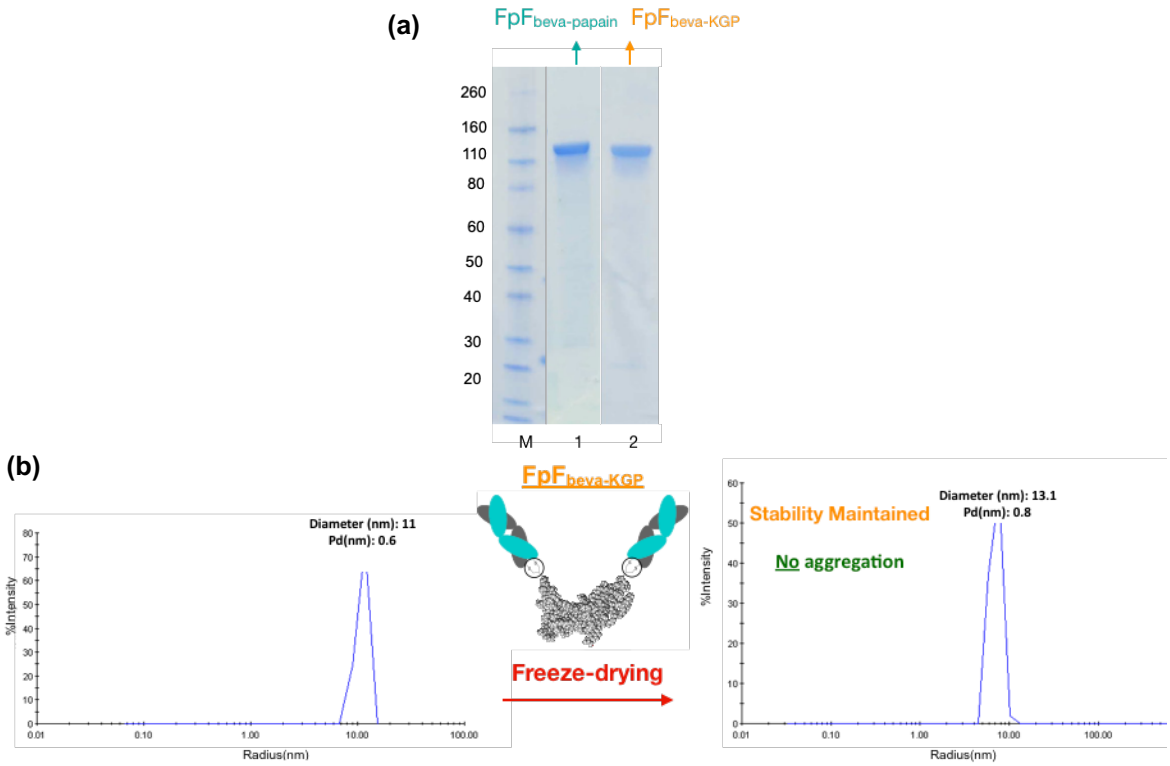


Figure 6. (a) Representative SDS-PAGE gels for the final purified $FpF_{beva\text{-}papain}$ and $FpF_{beva\text{-}KGP}$. Gels were stained with colloidal blue, Lane M; protein Standard, lanes 1, 2; purified $FpF_{beva\text{-}papain}$ and $FpF_{beva\text{-}KGP}$ respec tively appeared at about 120 kDa molecular weight band (2 x 50 kDa Fabs + 10 kDa PEG; PEG moves twice its molecular weight size; overall is 120 kDa). **(b)** DLS analysis for $FpF_{beva\text{-}KGP}$ before and after lyophilisation in PBS only buffer. $FpF_{beva\text{-}KGP}$ maintained its stability after being lyophilised in contrast to full IgG.

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