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Optimization of Enzymatic Antibody Fragmentation for Yield, Efficiency, and Binding Affinity

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Abstract

Enzymatic antibody fragmentation has been well studied for various hosts and isotypes, but fragmentation patterns also vary unpredictably by clone, and optimizing Fab or F(ab')₂ production by trial and error consumes large quantities of antibodies. Here, we report a systematic strategy for optimizing functional F(ab')₂ production via pepsin digestion from small quantities of IgG. We tested three key parameters that affect fragmentation: pH, enzyme concentration (% pepsin w/w), and reaction time, and found that pH had the greatest impact on fragmentation yield and efficiency. We then developed a systematic approach to obtaining acceptable yields, digestion efficiency, and binding affinity. Three case studies are described to illustrate the approach. We anticipate that this work will provide a quick and cost-effective method for researchers to produce antibody fragments from whole IgG, avoiding haphazard trial and error.

Graphical Abstract

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Supporting Information

Supporting figures for the identification of F(ab')₂ band fragments in non-reducing SDS PAGE and an example of mass quantitation in SDS PAGE using calibration curve. Supporting method for a preliminary experiment to an ELISA for affinity to find plate coating concentrations of antigen.

Conflict of interest statement

The authors have no conflicts of interest to report.

Introduction

Antibody fragments have enjoyed widespread use in a variety of disciplines ranging from bioanalytical immunoassays to pharmacological research. Their small size and lack of an Fc-mediated binding region allow for better penetration into tissue and specificity for numerous applications.^{1–4} There are two primary ways to produce such fragments: digestion with an enzyme such as papain for Fab fragments or pepsin for F(ab')₂ fragments,⁵ or expression of a recombinant antibody fragment. The latter requires either knowledge of the sequence of the antibody, or a lengthy process of production from a host animal and RNA transfer to *E. coli* through a bacteriophage.⁶ This work focuses on enzymatic digestion, as it rapidly produces fragments from commercially available antibodies.

Although enzymatic antibody fragmentation with pepsin has been studied since the 1960's and 70's, most laboratories still must optimize the fragmentation of each particular antibody by trial and error. Commercial kits for fragmentation are widely available, and these offer suggested digestion conditions based on reported trends for isotype and host species.^{7–12} However, reports of fragmentation as a function of clonal differences are anecdotal, which can necessitate optimization by the user for each clone. Furthermore, the success criteria for fragmentation may vary based on the intended use of the fragments. Yield, purity, or efficiency of the reaction are frequent criteria. Another is the retention of binding affinity; depending on the reaction conditions, enzymatic digestion can impair function through aggregation or denaturation.¹⁰ There is no one optimal procedure for fragmentation reactions because each antibody and reaction presents unique challenges and goals.

A systematic approach to antibody fragmentation is needed to accelerate progress in laboratories working to generate their own antibody fragments. Such a procedure may prevent optimization from devolving into a series of trial and error experiments, which can quickly become cost prohibitive due to high price of antibodies. In this paper we report the development of such an approach for fragmentation by pepsin, designed to identify a set of conditions that meets the acceptance criteria for yield, digestion efficiency, and function as

quickly as possible. We focused on digestion by pepsin to generate $F(ab')_2$ fragments, which offer a higher avidity than single Fab fragments. We systematically determined the importance of pH, amount of enzyme, and digestion time in producing functional fragments from small (50 – 1000 ug) quantities of commercially available antibodies. Three case studies were used to illustrate the method and to identify key factors to be aware of when optimizing.

Results and Discussion

Fragmentation yield and efficiency varies by clone under identical conditions

Here we focused on fragmentation of rat IgG antibodies because of their widespread use to detect antigens in murine tissues. Like other species of antibodies, rat IgGs differ in their sensitivity to pepsin depending on isotype ($IgG\ 2c > 2b > 2a > 1$), with less sensitive isotypes requiring longer digestion times, more enzyme, or lower pHs.⁸ However, these isotypic trends may not always translate to individual clones or preparations of antibody. Clonal factors such as primary sequence can determine how the antibody unfolds under acidic conditions, which may impact the efficiency of pepsin cleavage.¹³ In addition, the degree of glycosylation can vary based on host, isotype, and recombinant culture conditions, and the sterics of the glycans can alter the interaction between an antibody and the active site of pepsin.¹⁴

To illustrate this variation, we digested multiple rat IgGs with pepsin under identical conditions. Four clones used commonly for immunological experiments, spanning three isotypes, were selected for study: three clones of rat IgG1 (HRPN: isotype control, R4-6A2: anti-mouse IFN- γ , and XMG1.2: anti-mouse IFN- γ), a rat IgG2a (RA3-6B2: anti-mouse/human CD45R/B220), and a rat IgG2b (GK1.5: anti-mouse CD4). We tested a fragmentation condition that is typically recommended by commercial vendors: 1 mg/mL antibody at pH 4.4, using 125 % w/w pepsin for 2 hours.

Fragmentation followed patterns typical for most antibodies (Fig 1a). Residual unfragmented IgG appeared in the SDS-PAGE at ~ 140–160 kDa. Most of the antibodies ran as a doublet of peaks (except clone GK1.5), possibly due to multiple conformational states of the proteins (Fig 2b). This may be a result of only partially denaturing the samples with SDS and not performing a heat treatment and disulfide reduction of samples before gel analysis. Doublets were observed in undigested samples as well (data not shown). For most clones, the $F(ab')_2$ fragment appeared close to the canonical molecular weight of 90 – 100 kDa, with a partially digested product (likely loss of a single Fc heavy chain) at ~ 125 kDa. Even this could vary, however, as seen with clone GK1.5, whose $F(ab')_2$ appeared at ~ 80 kDa. The identity of the $F(ab')_2$ bands were confirmed in one of two ways: 1) treatment of the sample with 2-MEA reducing agent, which eliminated the $F(ab')_2$ band by reducing cysteine disulfides connecting the two Fab' regions, resulting in a 40–50 kDa band indicative of single Fab' fragments, or 2) passing the sample through an anti-Fc- γ affinity column, which removed incompletely digested products and retained the $F(ab')_2$ band (Fig. S1).

The SDS-PAGE data was used to quantify the yield (Eq. 1, Fig. 1d) of F(ab')₂, digestion efficiency (Eq. 2), and purity (Eq. 3) of the reaction:

$$\% \text{ yield of } F(ab')_2 = \frac{\text{mol of } F(ab')_2 \text{ produced}}{\text{mol of starting IgG}} \times 100 \quad (1)$$

$$\begin{aligned} \% F(ab')_2 \text{ Efficiency} \\ = \frac{F(ab')_2}{\text{Undigested (150 kDa)} + \text{Partially Undigested (125 kDa)} + F(ab')_2(100 \text{ kDa})} \times 100 \end{aligned} \quad (2)$$

$$\% F(ab')_2 \text{ Purity} = \frac{F(ab')_2}{\text{all products}} \times 100 \quad (3)$$

Efficiency is distinct from purity as a readout, as efficiency reports on the conversion of the intact IgG to F(ab')₂, whereas purity is concerned with the ratio of F(ab')₂ to all fragments produced. Efficiency was defined as the percent of F(ab')₂ product out of undigested and partially undigested products and purity was defined as the percent of F(ab')₂ out of the total products, including all of the smaller fragments (Fc fragments and other small over-digested/degraded fragments). Thus, conditions that have a high digestion efficiency could potentially have a significantly lower purity due to the inclusion of smaller fragments (Fig 1e–f). Whether purity or efficiency is the more relevant metric will depend on the intended use of the product. Here, we used efficiency because our goal was primarily to remove the Fc region from the antibody, and smaller fragments would not affect our downstream applications.

Under these conditions, both the yield and efficiency of digestion varied more than 10-fold between clones and isotypes of rat IgG. Even amongst the three IgG1s, F(ab')₂ yield ranged from 4.0 to 42 %, and efficiency between 9.2 and 99 %. These data clearly demonstrate the need for tailored reaction conditions for each antibody clone if high yield, efficiency, or purity is desired.

Fragmentation efficiency and yield are most sensitive to pH

There are multiple variables that affect the efficiency and yield of an enzymatic fragmentation reaction, including reaction time, temperature, pH, concentrations of antibody and enzyme, and mixing during reaction. For these experiments, we focused on three variables that had a significant impact in prior studies: pH, ratio of enzyme to antibody (% w/w), and reaction time.¹⁰ The reaction time and the ratio of enzyme (pepsin) concentration to substrate (antibody) concentration drive the reaction based on enzyme kinetics, whereas pH has a more indirect effect by impacting enzymatic activity and protein unfolding. Pepsin is most active at a pH of 2, and gradually loses activity as pH is increased until being

irreversibly denatured at a pH of 6.¹⁵ In addition, it is thought that as pH decreases, some antibodies may slightly unfold or denature, increasing the accessibility of the antibody to the pepsin's active site.

Because the three selected variables are not independent, small trial-and-error-based changes in reaction conditions can easily result in under or over-shooting the desired efficiency, with a trade-off in yield. For example, the optimal enzyme concentration likely increases at higher pH as the enzyme loses activity, so changing only the concentration or only pH is not ideal. In this situation, a systematic Design of Experiments approach proves especially useful to quickly identify the most critical parameters.¹⁶ Rather than vary one variable at a time, multiple variables are tested simultaneously to identify critical variables and co-dependencies.

Here, we used this systematic approach to determine which variable had the greatest effect on the % yield of the F(ab')₂ fragment and efficiency of the reaction. We fragmented a rat IgG1 isotype control (HRPN), an inexpensive and readily available antibody, and simultaneously tested all three variables with two levels each ($2^3 = 8$ combinations of conditions). The array of conditions tested was representative of oft-suggested conditions for antibody fragmentation: pH at 2.8 or 4.4, pepsin concentration at 1 or 20 %, and reaction time at 2 or 6 hr. The antibody concentration was kept constant at 1 mg/mL, and all reactions were conducted at 37 °C with continuous gentle agitation.

Over this range of conditions, a change in pH had the greatest impact, followed by change in concentration of pepsin (Fig. 2). Digestion at a low pH of 2.8 produced the highest yields and efficiency of HRPN F(ab')₂, reaching 99 % yield and 100 % efficiency at the optimal condition (Table 1). While it is possible that varying the pepsin concentration or time over a greater range (e.g. 1 % – 200% pepsin or 1 h – 24 h) may increase the impact of those variables, higher concentrations and times can lead to over-fragmentation and/or degradation of the antibody.¹⁷

Identification of acceptance criteria

Practically speaking, for most experiments, optimizing for the highest possible yield and efficiency is unrealistic and unnecessarily laborious. It is more useful to define in advance the “acceptance criteria” – the minimum values of yield and efficiency (and function) that are considered sufficient. The values of acceptance criteria will depend on the application. The requirement for % yield is likely driven by cost considerations and the quantity of the fragment that is required for downstream use. The required purity and efficiency of the reaction depends on both the intended use and how easily the products can be purified by subsequent processing. Finally, the requirement for functionality (i.e. binding affinity) depends on whether the fragment will be used for antigen binding, versus simply being sequenced.

Whether reaction efficiency is a priority likely depends largely on the available options for downstream purification. When working with small quantities of antibody (50 µg per reaction), isolation of functional F(ab')₂ fragments from other fragments may be challenging. Microscale size exclusion chromatography is the best option, but requires

specialized equipment.¹⁸ For many antibodies, affinity-based techniques such as proteins A and G, which bind to antibody Fc regions, may prove a suitable means to remove residual unfragmented or partially fragmented products from the mixture.¹⁹ For others, however, this approach is not viable. For example, protein A has little to no affinity for rat IgG. Protein G does have some affinity for rat IgGs, especially rat IgG2a, but it binds both the Fc and Fab regions of the antibody, compromising its use for purification.^{19–21} Thus, for easily purified antibodies, especially those available at large scale, a mediocre efficiency (~50–80%) may be acceptable, but for others a much higher efficiency is needed.

Case Studies: On reaching acceptable yield, efficiency, and function in real life

As a series of case studies, this approach to optimizing fragmentation was applied to three rat IgG's (Table 1). We intended to use the F(ab')₂ fragments as detection reagents in applications such as flow cytometry, ELISA, and immunofluorescent staining of tissue.³ Acceptance criteria were determined based on this set of applications as follows. Given that the selected clones were available in bulk from commercial vendors, we determined that a yield of > 50 % was sufficiently cost effective to accept. However, for some of the intended applications, residual Fc regions would be detrimental. Because of the difficulty of further purifying rat F(ab')₂ fragments, we prioritized reaction efficiency over yield, and initially required an efficiency of > 90%. Finally, because we intended to use the fragments for antigen binding, it was also critical that the binding affinity remain high, ideally within 2-fold of the IC50 of the original antibody as measured by an affinity ELISA. In each case, we first screened conditions for yield and efficiency, then confirmed the binding affinity of the product.

Case Study 1: First, we sought to produce F(ab')₂ fragments from anti-mouse CD4 (clone GK1.5), which is a rat IgG2a antibody. CD4 is a canonical phenotypic marker for helper T cells in flow cytometry and immunofluorescence. Based on the results shown above, in which pH had the most significant impact on the fragmentation, the pH was varied in three levels from 2.8, the optimal value for HRPN, and the traditional pH 4.4, which is recommended in many commercial kits. The % pepsin was varied simultaneously in two levels from 1 to 10%. Reaction time was held constant at a convenient value (2 hr).

Once again, we observed that pH had a substantial impact on yield and efficiency (Figure 3a–b), especially at lower pepsin concentration. Pepsin concentration also had a substantial impact, especially at higher pH, where it was presumably less active. Fortunately, one of the tested conditions (pH 3.5, 10% pepsin, 2 hours) surpassed the acceptance criteria for yield and efficiency (Table 1), and thus further optimization was not required. The F(ab')₂ product from that condition was subsequently tested for affinity, and was found to have comparable affinity to its whole antibody precursor (~15 nM IC50 for both; Figure 3c). Thus, this condition was accepted for further use. This case study demonstrates the usefulness of screening multiple pHs over a limited range of pepsin percentage and reaction time, which can quickly identify a near optimal condition that can be further refined if needed.

Case Study 2: We found that for some antibodies, despite modulating pH, time, and pepsin, it was difficult to obtain high yield and efficiency simultaneously. For example, this

phenomenon occurred with fragmentation of anti-mouse IFN- γ (clone R4-6A2), a rat IgG1 antibody. IFN- γ is a cytokine, i.e. a protein involved in extracellular signaling between immune cells, that is critical for adaptive immunity. In attempting to optimize this antibody, we tested pHs of 3.5, 4.0, and 5.0 with a wide range of pepsin concentrations and reaction times, in an extensive series of optimization experiments. We observed that a pH of 5.0 produced the greatest yields, and at lower pH such as pH 2.8 the antibody denatured and aggregated (data not shown). At pH 5.0, yields were never above 50 %, and increasing the efficiency by increasing the pepsin concentration or time often came with a further tradeoff in yield due to over-fragmentation (Figure 4a,b). Given our acceptance criteria and priorities outlined above, the most suitable condition was pH 5.0 with 100 % pepsin w/w for 24 hours, which had the highest efficiency but the lowest yield (Table 1). When the F(ab')₂ fragment from that condition was tested for affinity, its IC₅₀ was comparable to the affinity of whole (unfragmented) antibody (Figure 4c), so this condition was accepted for further use. This case study demonstrates the need to compromise between acceptance criteria for some antibodies.

Case Study 3: As a final case, we generated F(ab')₂ fragments from anti-mouse IFN- γ (clone XMG1.2), a rat IgG1 antibody. This antibody forms an ELISA pair with R4-6A2 for detection of IFN- γ . Based on the results above, we initially screened several conditions at pH 2.8 and 4.4 (Figure 5a,b). Digestion at pH 2.8 resulted in denaturation and aggregation of antibody and thus was unable to be quantified. A pH of 4.4 produced high efficiency with 50% pepsin, but yield was lower than desired. Therefore, in an effort to increase yield, we kept the 50% pepsin concentration, raised the pH to 5.0, and screened reaction time as a third variable (Figure 5d,e). This increased the yield with minimal loss of efficiency. Interestingly, in both scenarios, the apparent affinity of the F(ab')₂ fragment was ~50-fold higher than that of the intact antibody (Figure 5c,f). One potential explanation may be that the removal of the Fc region increases the flexibility of the hinge between the Fab arms, reducing steric strain and improving bivalent binding.²² In summary, this case study indicates that even small modulations in pH can increase yield, especially for antibodies that aggregate at low pH, and that F(ab')₂ fragments can sometimes have improved affinity over their parent IgG.

Summary: Suggested optimization procedure

Given the wide range of possible conditions for fragmentation, it is reasonable to ask where one should start when working with a new antibody. If resources are abundant, then a thorough initial screen could be performed over a wide range of conditions (Figure 6), using Design of Experiments to reduce the number of conditions tested. However, often both time and reagents are precious. If a simple first experiment is desired, based on our experience with the antibodies above, we suggest the following four conditions: pH (2.8 and 4.4) x pepsin (10 % and 50 %), with a reaction time of 2 hours. Afterwards further optimization can be performed if the acceptance criteria are not met, using the results of the first screen to point to the next set of conditions. After screening, once a condition with suitable yield, efficiency, and affinity is found, further minor changes to the condition can be made to “dial in” on the exact specifications desired. For example, one could increase digestion time by 10–30 minutes to boost digestion efficiency by 5–10 % depending on exact conditions used.

Conclusions

In this work we have outlined a simple yet systematic strategy to reach desired levels of yield, efficiency, and function of F(ab')₂ fragments from small quantities of IgG, including analysis of affinity after enzymatic digestion. We determined that optimization over a range of pHs has the largest impact, likely because it directly impacts the activity of pepsin and the steric constraints of interaction with the active site of the enzyme. pH screening may be followed by or conducted in parallel with optimizing the ratio of pepsin to the antibody. Reaction time had only a small impact and did not always require optimization. This process can be re-iterated until desired yield, efficiency, and function of F(ab')₂ fragments is achieved. This general outline for fragmentation has been performed successfully for several antibodies used routinely in our lab. We envision that this work will provide a quick and cost-effective guide for researchers to produce in-house generated antibody fragments, without the need for recombinant expression or haphazard trial and error approaches.

Experimental Procedures

Filter Preparation procedure

The following procedures used Amicon Ultra 0.5-mL 50-kDa molecular weight cut off (MWCO) centrifuge filters (Fisher Scientific, Waltham, MA) for concentration and buffer exchanges of antibody samples. In preliminary work, we found that PEO-coating was critical to prevent loss of protein to the filter when working with small quantities of antibody; we estimated that up to 10 µg of antibody was lost to an unblocked filter. Therefore, the Amicon filters were coated with 100 kDa polyethylene oxide (PEO) (Fisher Scientific) before use.²³ PEO solution (50 mg/mL) was centrifuged in the filter at 14,000 x g for 3 min, then manually rinsed out with DI water. The filters were then centrifuged with DI water for 14,000 G for 3 min, then inverted and centrifuged at 1,000 G for 1 min before use.

Antibody fragmentation procedure

The antibody clones used were HRPN, R4-6A2, XMG1.2, GK1.5 (Bio X Cell, West Lebanon, NH), and RA3-6B2 (BioLegend, San Diego, CA). Antibodies were buffer exchanged to the desired pH by using a PEO-treated 50-kDa MWCO centrifuge filter to put them into 100 mM formic acid buffer (pH 2.8 – 3.5) or 20 mM sodium acetate buffer (pH 4.0 – 5.0). These buffers were made in house with formic acid or sodium acetate in DI water and adjusting the pH with HCl or NaOH. All reagents were from Fisher Scientific unless otherwise noted.

Antibodies were fragmented using immobilized pepsin on 6 % agarose resin (pepsin resin, Fisher Scientific). This pepsin product was reported to have 1–1.5 mg/mL enzyme in a mix containing glycerol storage buffer. The same batch of pepsin was used for all samples (LOT: SB246536, activity 10,012 Units/mL). To prepare the pepsin resin for use, it was removed from storage buffer, pipetted into a 0.5 mL Peirce spin column (Fisher Scientific), centrifuged for 30 s at 100 G, and resuspended in the desired pH buffer (i.e. formic acid buffer or acetate buffer). Next, the immobilized pepsin and the buffer-exchanged antibody solution were mixed together so that the final concentration of antibody was 1 mg/mL. The

sample was placed in an incubator at 37 °C on a shaker, shaking gently for the duration of the reaction. After the reaction, fragmented antibody sample was separated from immobilized pepsin using a 0.5-mL Peirce spin column, which allows the antibody solution to pass through while retaining the resin-bound enzyme above the column. The immobilized pepsin resin was rinsed several times with phosphate buffered saline (PBS, no calcium or magnesium). The sample (eluent from the first and subsequent rinses) was collected, then buffer exchanged into PBS and concentrated using a PEO-coated 50-kDa MWCO centrifuge filter. F(ab')₂ fragments were confirmed (see Supplementary Fig. S1) via reduction of cysteine disulfide bonds by beta mercaptoethylamine (2-MEA, Fisher Scientific) or affinity purification using anti-Fcγ antibody (Jackson ImmunoResearch, West Grove, PA). Antibody samples were stored at 4°C in PBS until analysis.

SDS PAGE and analysis of data

Antibody fragmentation samples were analyzed using 4–12 % Bis Tris NuPAGE SDS PAGE (Fisher Scientific) with MOPS buffer (Fisher Scientific). To preserve the structure of antibodies and F(ab')₂ during analysis, the samples were not reduced (no beta-mercapto ethanol) and were not heat-treated, unlike standard treatment prior to SDS PAGE. In addition to the fragmentation samples and controls, a standard curve (1 mg/mL – 0.1 mg/mL) of corresponding whole antibody was also run on the gel. A Precision Plus molecular weight ladder (BioRad, Hercules, CA) was included in lane 1. Samples were prepared according to vendor specifications using Lithium dodecyl sulfate (LDS) sample buffer 4X (Fisher Scientific) and DI water. Sample loading was calculated to never exceed 1.5 µg/lane. SDS PAGE was run at 175 V constant for 52 min, which was long enough for the solvent front to reach the end of the gel. Gels were stained with Commassie R-250 (Fisher Scientific) as per vendor instructions.

Images of gel were collected on a ChemiDoc XRS+ (BioRad), and images were analyzed using Image Lab v5.2.1 (BioRad). Using Image Lab, the integrated stain intensity (area under the curve, AUC) was found for each protein band, and background subtraction and peak selection were performed uniformly across all samples within the same gel (Figure S2). Using the standard curve and molecular weight ladder, mass and molar concentrations of each band were calculated, as well as % yield (Eq 1). Digestion efficiency (Eq 2) and purity (Eq 3) of F(ab')₂ were calculated using peak areas. When samples were run in duplicate on the gel, the average AUC was used for analysis.

ELISA for affinity

An ELISA was performed to measure the affinity of antibody fragments for their target protein antigen, as compared to the affinity of whole antibody precursors, by following the procedure outlined in published protocols.^{24,25} In this assay, a fixed concentration of antibody was equilibrated with antigen in solution, with the antigen at concentrations ranging from below to above the IC₅₀ of binding, and then the mixture was added to a plate coated with the antigen. Antibody that bound to the plate was detected using a labelled secondary antibody. This assay design produces a negatively-sloped sigmoidal curve whose midpoint reports the IC₅₀ of the antibody-antigen interaction. IC₅₀ is expected to be similar

to the Kd for the low coating concentrations of antigen used here (SI methods). The detailed procedure was as follows:

F(ab')₂ samples from fragmentation were visually confirmed to be > 98% free of products that contained an Fc region by non-reducing SDS-PAGE before affinity analysis (i.e., > 98 % efficiency). The antigens used were recombinant mouse interferon gamma (IFN-γ, Peprotech, Rocky Hill, NJ) and soluble mouse CD4 protein-his tag (Sino Biological, Wayne, PA). The general procedure was as follows: Serial dilutions of antigen (0 – 256.4 nM, in 2-fold or 5-fold dilutions) were prepared in 1 % BSA and 0.05 % Tween-20 (Fisher Scientific) in PBS (block solution), and then mixed 1:1 v/v with a constant concentration (6.67 nM final concentration) of antibody in block solution (mixed sample). Mixed samples were stored overnight at 4 °C. Separately, the antigen was coated onto a high-binding plate (Corning Costar 96 well 1/2 area, #3690; Fisher Scientific) in PBS overnight at 4°C (IFN-γ at 1–3 μg/mL, CD4 0.2 μg/mL), after which the wells were blocked for 1 hour with block solution. The mixed samples were added to the plate and incubated for 2 hours, then washed. Horseradish peroxidase enzyme (HRP) - conjugated goat anti-rat F(ab')₂ secondary antibody (Jackson ImmunoResearch) was added to the plate at 0.5 μg/mL in block solution. All washing steps were performed with 0.05% Tween-20 in PBS. Plates were developed using TMB substrate (Fisher Scientific) and absorbance values were read at 450 nm on a plate reader (CLARIOstar; BMG LabTech, Cary, NC). Sample curves were fit in GraphPad Prism 6 with a sigmoidal 4 parameter curve (Eq 4), where X is log of antigen concentration, Y is absorbance, min and max are the plateaus of the sigmoidal curve on the Y axis, and HillSlope describes the steepness of the slope.

$$Y = \min + \frac{\max - \min}{1 + 10^{(\text{Log}(\text{IC50} - X) * \text{HillSlope})}} \quad (4)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antibodies of different clones, regardless of isotype, can fragment differently under the same conditions. (a) Reaction scheme for pepsin digestion. The pictured fragments are the most likely products for the bands observed at those molecular weights, for a canonical 150 kDa antibody. (b) Non-reducing SDS PAGE of antibodies digested at pH 4.4 with 125% Pepsin (% w/w) for 2h at 37°C. N=1 per condition and were ran in duplicate on gel (two lanes per condition). Lane contents are listed above each lane. Molecular weights (MW, kDa) of the ladder in Lane 1 are listed at the left. Note that for GK1.5, $F(ab')_2$ appears at a non-canonical weight of 80 kDa. (c) To quantify the fragmentation pattern, the relative mass % of all sample bands were quantified using a gel imager. (d) % yield, (e) digestion efficiency, and (f) the purity of $F(ab')_2$ produced the tested conditions.

Figure 2.

A change in pH had the greatest effect on yield and efficiency of F(ab')₂ production by pepsin. Rat IgG1 clone HRPN was fragmented under 2³ = 8 conditions of pH, % pepsin (% w/w), and time. (a) % molar yield of F(ab')₂ produced in each condition. (b) The change in yield as a result of increasing pH, % pepsin, or time. (c) % efficiency of reaction in each condition. (d) The change in efficiency as a result of increase pH, % Pepsin, or time. N=1 for each reaction condition. Conditions were measured twice on SDS PAGE.

Figure 3.

Case Study 1: pH had the greatest effect on F(ab')₂ yield for anti-CD4 clone GK1.5. (a) % molar yield and (b) efficiency of F(ab')₂ produced during fragmentation. N=1 per condition. The condition marked with blue square was tested by (c) indirect ELISA to measure affinity of F(ab')₂ fragment as compared to whole antibody. IC₅₀: F(ab')₂ - 14.5 nM; Whole - 15.6 nM. N=3 per condition. Data was fit with 4 parameter curve in GraphPad Prism.

Figure 4.

Case study 2: For one antibody, the yield of F(ab')₂ was inverse with respect to efficiency. (a) % molar yield and (b) efficiency of F(ab')₂ fragment production for anti-IFN- γ clone R4-6A2 at pH 5.0. N = 1 per condition. The condition marked with a blue square was tested by (c) indirect ELISA to measure the affinity of the F(ab')₂. IC₅₀ for F(ab')₂ - 7.4 nM; Whole - 10.4 nM. N=3 per condition, data was fit with 4 parameter curve in GraphPad Prism.

Figure 5.

Case study 3: Yield and efficiency can vary with pH. Digestion of anti-IFN-γ clone: XMG1.2 at pH 2.8 and pH 4.4 has (a) % molar yield and (b) efficiency of F(ab')₂ produced (* aggregation/ unable to be quantified). (c) affinity ELISA of best condition (marked with blue square). IC₅₀: F(ab')₂ - 0.25 nM; Whole - 20.4 nM. Digestion at pH 5.0 shows increased (d) yield and (e) efficiency of F(ab')₂ produced, and has maintained affinity as measured by (f) affinity ELISA (tested condition marked with red square). IC₅₀: F(ab')₂ - 0.41 nM; Whole - 23.0 nM. N=1 per fragmentation condition and ran in duplicate on SDS PAGE. N=3 per indirect ELISA condition, data was fit with 4 parameter curve in GraphPad Prism.

Figure 6.

Suggested approach to optimization of fragmentation by pepsin. Each column represents one variable for optimization. Ideally, at least pH and % pepsin should be optimized simultaneously. The values displayed on each column represent its normal range for fragmentation. Highlighted in gray dashed boxes are conditions suggested for a simple first experiment: pH (2.8 and 4.4), Pepsin (10% and 50%), and a time of 2 or 6 hours. Afterwards further testing can be performed if needed to meet acceptance criteria.

Table 1:

Table of optimized digestion conditions and results

Clone	Isotype	Antigen	Apparent F(ab') ₂ MW	pH	Time (h)	% Pepsin (w/w)	Yield	Efficiency	IC50 (nM) of F(ab') ₂
HRPN	IgG1	α-HRP	100	2.8	6	1	99.2	99.4	-
GK1.5	IgG2b	α-mouse CD4	80	3.5	2	10	100	91.8	14.5
R4-6A2	IgG1	α-mouse IFN-γ	100	5.0	24	100	14.7	99.8	7.4
XMG1.2	IgG1	α-mouse IFN-γ	100	5.0	4.5	50	67.8	97.9	0.41

- Indicates that sample was not tested/measured