Research Article

The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: Down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms

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The tobacco-related species *Nicotiana benthamiana* has recently emerged as a promising host for the manufacturing of protein therapeutics. However, the production of recombinant proteins in *N. benthamiana* is frequently hampered by undesired proteolysis. Here, we show that the expression of the human anti-HIV antibodies 2F5, 2G12, and PG9 in *N. benthamiana* leaves leads to the accumulation of discrete heavy chain-derived degradation products of 30–40 kDa. Incubation of purified 2F5 with *N. benthamiana* intercellular fluid resulted in rapid conversion into the 40-kDa fragment, whereas 2G12 proved largely resistant to degradation. Such a differential susceptibility to proteolytic attack was also observed when these two antibodies were exposed to various types of proteinases in vitro. While serine and cysteine proteinases are both capable of generating the 40-kDa 2F5 fragment, the 30-kDa polypeptide is most readily obtained by treatment with the latter class of enzymes. The principal cleavage sites reside within the antigen-binding domain, the V_H–C_H1 linker segment and the hinge region of the antibodies. Collectively, these results indicate that down-regulation of endogenous serine and cysteine proteinase activities could be used to improve the performance of plant-based expression platforms destined for the production of biopharmaceuticals.

Supporting information available online

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Abbreviations: BSA, bovine serum albumin; **CDR,** complementarity-determining region; **CHO,** Chinese hamster ovary; **ER,** endoplasmic reticulum; **IgG,** immunoglobulin G; **LC,** liquid chromatography; **mAb,** monoclonal antibody; **MS,** mass spectrometry; **PAGE,** polyacrylamide gel electrophoresis; **PBS,** phosphate-buffered saline; **PMSF,** phenylmethylsulfonyl fluoride; **SDS,** sodium dodecyl sulfate

1 Introduction

Due to their outstanding specificities, monoclonal antibodies (mAbs) have become one of the most popular products of the biopharmaceutical industry. In recent years, plants have gained increasing attention as versatile expression platforms for recombinant therapeutic proteins such as mAbs [1]. Due to the large extent of similarity between the secretory pathways of plants and mammals, even highly complex molecules like mAbs can be efficiently produced in plant systems [2]. The possibility

to produce grams of purified mAbs within days after generation of an appropriate DNA construct [3] provides unique advantages for plant-based expression platforms when production speed is of utmost importance, as necessary for the generation of individualized cancer vaccines [4].

Recent advances in the development of transient expression systems have placed *Nicotiana benthamiana* in a particularly favorable position since this tobacco-related plant species is well suited for the large-scale production of therapeutic proteins. Nevertheless, a major problem encountered with recombinant protein production in *Nicotiana* species remains to be solved: the proteolytic degradation of the target protein within the plants [5, 6]. Recent studies have shown that co-expression of proteinase inhibitors is a promising approach to alleviate unwanted proteolysis in plant cells and whole plants [7, 8]. Alternatively, down-regulation of endogenous proteinase activities by means of RNA interference has been attempted for improvement of the performance of plant-based expression platforms destined for the production of protein therapeutics [9]. For either strategy, substantial knowledge about the host enzymes involved in proteolytic breakdown of foreign proteins is required [10], but genetic and biochemical information on *N. benthamiana* proteinases is still scarce [11]. Alternatively, characterization of the cleavage sites within the protein of interest can provide hints about the proteinases involved in its degradation. Unfortunately, only one such cleavage site has been elucidated so far for mAbs produced in plants [12].

In this study, we have performed a detailed characterization of the degradation fragments observed upon expression of the three anti-HIV mAbs 2F5, 2G12, and PG9 [13–15] in *N. benthamiana*. Furthermore, the proteolytic susceptibility of 2F5 and 2G12 was tested in vitro with a series of representative proteinases. Collectively, these results suggest that mAb proteolysis in *N. benthamiana* is largely due to serine and cysteine proteinases.

2 Materials and methods

2.1 Construction of mAb expression vectors

The MagnICON expression vectors pICH26033 and pICH31160 (kindly provided by Viktor Klimyuk, Icon Genetics, Halle, Germany) were modified by insertion of the coding sequence for the signal peptide of barley α-amylase, yielding the plasmids pICHα26033 and pICHα31160. Codon-optimized PG9 heavy and light chain cDNAs (GeneArt, Regensburg, Germany; see Table S1 in Supporting information for protein sequences) were cloned with or without a C-terminal KDEL tag into the *Bsa*I sites of pICHα31160 and pICHα26033, respectively. The resulting vectors pPG9HC, pPG9HC-KDEL, pPG9LC, and pPG9LC-KDEL were first transformed into *Escheri-*

chia coli and then, after sequence confirmation, into the *Agrobacterium tumefaciens* strain GV3101::pMP90. The constructs used for the expression of 2F5, 2F5-KDEL, and 2G12 have been described in previous studies [16, 17]. All mAbs are human immunoglobulin G (IgG) antibodies of subclass IgG1 with either κ (2F5, 2G12) or λ (PG9) light chains.

2.2 mAb expression in *N. benthamiana*

N. benthamiana ΔXTFT plants lacking plant-specific α1,3-fucosylation and β1,2-xylosylation were grown at 24°C with a 16-h light:8-h dark photoperiod. Four- to five-week-old plants were used for agroinfiltration experiments as described previously [18]. Briefly, overnight cultures were pelleted and then resuspended in infiltration buffer (25 mM Mes buffer (pH 5.5), 25 mM $MgSO_4$, 0.1 mM acetosyringone) at an OD_{600} of 0.2 (1.0 OD_{600} corresponds to 5×10^8 cells/mL). In the case of PG9 expression, equal amounts of the strains carrying the respective heavy and light chain constructs were used. Infiltrated *N. benthamiana* leaves were harvested after 3 days.

2.3 Preparation of leaf extracts and intercellular fluid

For total leaf extracts, 250 mg fresh material was snap-frozen in liquid nitrogen and then ground in a ball mill (Retsch, Haan, Germany). After addition of 500 μL of extraction buffer (100 mM sodium acetate (pH 5.5), 40 mM ascorbic acid), the samples were incubated for 10 min at 4°C prior to centrifugation (5 min, 14 000*g*, 4°C) to remove insoluble material. In some experiments, the extraction buffer was supplemented with a proteinase inhibitor cocktail (P9599; Sigma–Aldrich, St. Louis, MO). For the recovery of intercellular fluid, fresh leaves were submerged in extraction buffer prior to vacuum exposure in a desiccator. The leaves were then blotted dry before centrifugation for 15 min at 1000*g* and 4°C. The recovered solution was concentrated by ultrafiltration. The total protein content of leaf extracts and intercellular fluid was determined with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as a standard.

2.4 mAb purification

Small-scale antibody purification was performed essentially as described previously [17]. Briefly, frozen leaf material was first crushed in a ball mill as above and then extracted with 2 μL buffer (45 mM tris/HCl (pH 7.4), 1.5 M NaCl, 40 mM ascorbic acid, 1 mM EDTA) per mg of leaf material for 15 min at 0°C. After centrifugation (5 min, 14 000*g*, 4°C), the supernatant was incubated with rProtein A Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, UK) for 90 min under constant agitation at 4°C. The

beads were then collected by centrifugation and washed with phosphate-buffered saline (PBS) prior to elution of bound antibody with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer.

2.5 mAb treatment with proteinases

Chinese hamster ovary (CHO)-derived mAbs 2F5 or 2G12 were provided by Polymun Scientific GmbH (Klosterneuburg, Austria) and tested for their susceptibility to the following proteinases: recombinant human cathepsin B [19]; recombinant human cathepsin L ([20]; from John S. Mort, Shriners Hospital for Children, Montreal, Canada); cathepsin D (from bovine spleen), chymotrypsin, subtilisin, trypsin (all from Sigma–Aldrich); recombinant human legumain ([21]; from Hans Brandstetter, University of Salzburg, Austria). In general, 2F5 or 2G12 (200 µg/mL) were treated with the respective proteinase (10–250 µg/mL) in 100 mM sodium acetate (pH 5.5) at 37°C. mAb digests with cysteine proteinases were performed in the presence of 2 mM cysteine. In the case of cathepsin D, 10 µM E-64 and 1 mM phenylmethylsulfonyl fluoride (PMSF) (both from Sigma–Aldrich) were added to inhibit contaminating cysteine and serine proteinases. Assays with total soluble leaf extracts or intercellular fluid were done in 50 mM sodium acetate (pH 5.5) or 20 mM sodium citrate/40 mM sodium phosphate (pH 4.0–7.0) at 37°C in the absence or presence of selected proteinase inhibitors (all from Sigma–Aldrich). After incubation for up to 16 h, reactions were stopped by addition of SDS–PAGE sample buffer.

2.6 SDS–PAGE and immunoblotting

mAbs and their fragments were fractionated by 12.5% SDS–PAGE. The gels were then either stained with Coomassie Brilliant Blue R-250 or blotted on nitrocellulose membranes (GE Healthcare). After blocking for 1 h in PBS containing 3% BSA, the membranes were incubated with anti-human IgG (γ-chain-specific; Sigma–Aldrich) or anti-human IgG ($C_H + C_L$-specific; Promega, Madison, WI) conjugated to horseradish peroxidase (HRP) at a concentration of 0.2–0.5 µg/mL PBS containing 0.05% Tween 20 (PBST) and 0.5% BSA for 90 min prior to development using chemiluminescence reagents (Bio-Rad). Monoclonal anti-KDEL antibodies (Merck Millipore, Darmstadt, Germany) were used at a concentration of 0.2 µg/mL in PBST containing 3% BSA and detected with anti-mouse IgG-HRP (Jackson ImmunoResearch, West Grove, PA) as outlined above.

2.7 Cleavage site analysis

N-terminal sequence analysis of bands blotted on polyvinylidene difluoride membranes (Bio-Rad) as described previously [22] was performed by Edman degradation on an Applied Biosystems Procise 492 protein sequencer (Protein Micro-Analysis Facility, Medical University of Innsbruck, Austria). The N-termini of mAb degradation products were also characterized by liquid chromatography (LC)-electrospray ionization–MS/MS in a similar way as reported earlier [23]. Briefly, mAbs and their fragments were separated by SDS–PAGE. The heavy-chain band degradation products were excised, S-alkylated and subjected to tryptic or chymotryptic digestion. Peptides were eluted from the gel slices with 50% acetonitrile, concentrated in vacuo and then separated by nano-LC (150 mm × 0.32 mm BioBasic-18; Thermo Scientific, Waltham, MA) with a gradient of 1–80% acetonitrile. Data mining was conducted in positive ion mode on a maXis-4GQ-TOF mass spectrometer (Bruker, Billerica, MA). MS2 scans of dominant precursor peaks were acquired and manually analyzed with DataAnalysis software version 4.0 (Bruker).

3 Results

3.1 Expression of mAbs 2F5, 2G12, and PG9 in *N. benthamiana*

The human anti-HIV antibody 2G12 could be produced in *N. benthamiana* without observing substantial amounts of degradation products as contaminants (Fig. 1A). These results are in agreement with our previous studies on this mAb [18], although N-terminally truncated heavy-chain fragments have been detected in 2G12 preparations derived from other plant species [17, 24, 25]. However, the production of the anti-HIV mAbs 2F5 and PG9 in *N. benthamiana* was associated with more pronounced proteolysis. In either case, a 40-kDa fragment was found to co-purify with intact heavy and light chains, which was less abundant in 2G12 samples. In sporadic cases of reduced light-chain synthesis, 2F5 preparations contained a major 30-kDa polypeptide (Fig. 1A, lane 1). The 40- and 30-kDa degradation products were both recognized by antibodies to the heavy chain, indicating that they represent truncated forms of this mAb component (Fig. 1A). In contrast, degradation products derived from the light chain were not observed. Notably, supplementation of the extraction buffer with a proteinase inhibitor cocktail did not prevent proteolysis (Fig. 1B). However, 2F5 and PG9 degradation was reduced upon addition of the endoplasmic reticulum (ER)-retrieval sequence KDEL to the C-termini of their heavy and light chains (Fig. 1A). It has been reported previously that the stability of antibodies *in planta* can be increased by retention of the proteins in this compartment [16, 26].

To characterize the degradation products in more detail, the N-terminal sequences of the 30- and 40-kDa fragments were determined by Edman degradation or mass spectrometry (MS). The 2F5 30-kDa fragment was
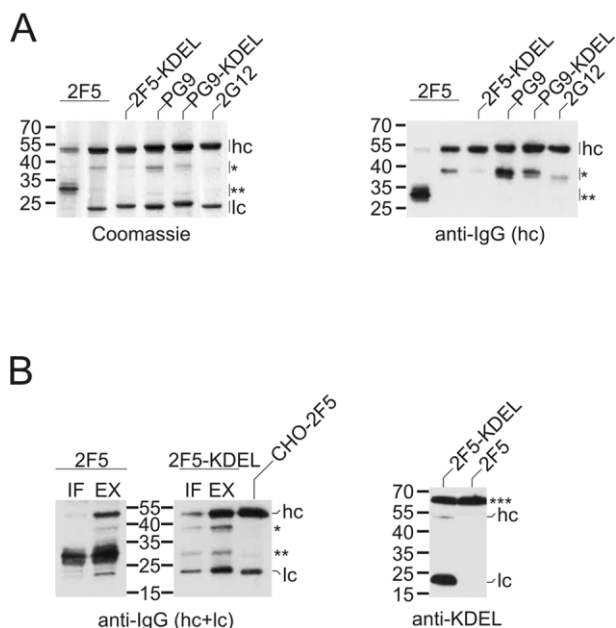
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Figure 1. Expression of 2F5, 2G12, and PG9 in *N. benthamiana*. (**A**) mAbs were affinity-purified from total soluble leaf extracts (30 mg leaf wet weight) and then analyzed by SDS–PAGE/Coomassie blue staining (left panel) or immunoblotting with antibodies to the heavy chain of human IgG (right panel). (**B**) Left panel: mAbs affinity-purified from intercellular fluid (IF; 100 mg leaf wet weight) or total soluble leaf extracts prepared in the presence of proteinase inhibitors (EX; 50 mg leaf wet weight) were analyzed by immunoblotting with antibodies to the heavy and light chains of human IgG. CHO-derived 2F5 (100 ng) was loaded as control. Right panel: total soluble extracts (2 μg protein) of leaves infiltrated with different 2F5 constructs were analyzed by immunoblotting with anti-KDEL antibodies (right panel). hc, heavy chain; lc, light chain; *40-kDa fragment; **30-kDa degradation product. Endogenous *N. benthamiana* BiP (***) served as internal control in the case of KDEL detection. The migration positions of selected molecular mass standards are indicated, with their respective masses expressed in kDa. The results shown are representative of at least two independent experiments.

found to be the result of a large N-terminal truncation since its *N*-terminus was identified as K^{247} (75%) or K^{248} (25%). This revealed that the main cleavage site (KVD^{246}↓K^{247}KVEP) was a few residues upstream of the hinge region connecting the C_H1 and C_H2 domains (see Table S1 of Supporting information for mAb heavy and light chain sequences). In the case of the 40-kDa fragment, cleavage occurred within the complementarity-determining region (CDR) H3 loop of the V_H domain, yielding predominantly G^{108} as *N*-terminal amino acid (TLF^{107}↓G^{108}VPIA). However, it should be noted that molecules starting with the next residue (V^{109}) were also found. The PG9 heavy chain was mainly cleaved within its CDR H3 loop (NYY^{111}↓D^{112}FYDG or YYD^{112}↓F^{113}YDGY), whereas the cleavage site identified within 2G12 was C-terminal to this structural element (GPG^{116}↓T^{117}VVTV).

To test whether the apoplast contains mAb degradation products, intercellular fluid was isolated from leaves infiltrated with 2F5 or 2F5-KDEL constructs. Comparison with total leaf extracts revealed that the fractions of the heavy-chain fragments and full-length mAb molecules accumulating in this compartment were similar (Fig. 1B). Interestingly, secretion of 2F5-KDEL was at best marginally lower than that of its counterpart lacking an ER retrieval sequence. Hence, we tested the heavy and light chains of 2F5-KDEL for the presence of the tag by means of immunoblotting with anti-KDEL antibodies. Although a specific signal could be obtained for both polypeptides, the heavy chain reacted much weaker than the light chain. This indicates a much higher proteolytic vulnerability of the targeting sequence when attached to the C-terminus of the heavy chain and suggests that this selective loss of the ER retrieval motif probably accounts for the inefficient intracellular retention of 2F5-KDEL (Fig. 1B).

3.2 Differential sensitivity of 2F5 and 2G12 to *N. benthamiana* proteinases

To test for the presence of mAb-degrading activities in *N. benthamiana* tissues, CHO-derived 2F5 and 2G12 were incubated in vitro with total soluble leaf extracts or intercellular fluid prepared from non-infiltrated plants. In the case of 2F5, a 40-kDa heavy-chain fragment was rapidly generated, in particular upon treatment with intercellular fluid (Fig. 2). In some instances, further processing into a 30-kDa fragment was also detected (data not shown). In contrast, 2G12 proved largely resistant to leaf proteinases under the same experimental conditions (Fig. 2). Similar results were obtained with intercellular fluid prepared from leaves infiltrated with *A. tumefaciens* carrying an expression vector for an unrelated recombinant protein (human transferrin) or the parental bacterial strain, indicating that the observed proteolysis is due to constitutively expressed plant proteinases (Supporting information, Fig. S1). The 2F5-hydrolyzing activity present in intercellular fluid was further characterized with respect to its pH profile and cleavage site. 2F5 heavy-chain processing into the 40-kDa form was strongest at pH 5.5–6.0, which conforms to the apoplastic pH in situ (Fig. 3A; Supporting information, Fig. S2A). To determine its *N*-terminal sequence, the 40-kDa degradation product was affinity-purified and then subjected to MS analysis. The most *N*-terminal peptide found was half-tryptic, commencing with residue S^{132}. Hence, cleavage occurred within the linker segment connecting the V_H and C_H1 domains (TIS^{131}↓S^{132}TSTK) and not in the CDR H3 loop as observed *in planta*. We also tested the effect of various synthetic proteinase inhibitors on 2F5 processing by *N. benthamiana* proteinases in vitro. 2F5 proteolysis by total soluble leaf extracts and intercellular fluid could be inhibited by addition of PMSF, a serine proteinase inhibitor that also displays some reactivity with cysteine proteinases. In contrast, inhibitors of aspartic and metalloproteinases
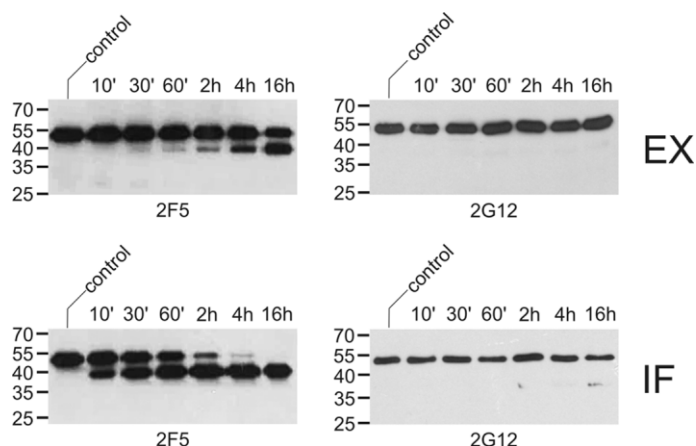
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Figure 2. Processing of 2F5 and 2G12 by *N. benthamiana* proteinases in vitro. CHO-derived 2F5 or 2G12 (400 ng) was incubated with intercellular fluid (IF; 650 ng protein) or total soluble leaf extracts (EX; 600 ng protein) for the indicated times and then analyzed by immunoblotting with antibodies to the heavy chain of human IgG. Untreated antibody was loaded as control. The migration positions of selected molecular mass standards are indicated, with their respective masses expressed in kDa. The results shown are representative of at least three independent experiments.

(pepstatin, phenanthroline) as well as trypsin- and papain-like enzymes (leupeptin, E-64) did not exert detectable effects on 2F5 fragmentation. Interestingly, addition of a commercially available proteinase inhibitor cocktail designed for prevention of unwanted proteolysis in plant extracts proved less effective than treatment with

PMSF. In this context, it should be pointed out that this proteinase inhibitor cocktail does not contain PMSF (Fig. 3B; Supporting information, Fig. S2B).

3.3 Susceptibility of 2F5 and 2G12 to representative proteinases

To get a better picture of which type(s) of proteinases could be involved in mAb degradation *in planta*, CHO-derived 2F5 and 2G12 were treated with representatives of different classes of proteolytic enzymes under conditions mimicking the milieu in the apoplast (pH 5.5). The cysteine proteinase papain is frequently used to prepare antibody fragments [27]. However, commercial papain can be contaminated with other papain-like cysteine proteinases displaying different specificities [28]. We therefore decided to test purified recombinant versions of two human papain-like cysteine proteinases, cathepsin B, and cathepsin L, for their ability to digest 2F5. Cathepsin L quickly converted the 2F5 heavy-chain into a 40-kDa fragment, which was further processed into a 30-kDa polypeptide upon prolonged incubation. Cathepsin B was much slower in generating the 40-kDa degradation product while formation of the 30-kDa form was not detected at all (Fig. 4). Analysis of either 40-kDa fragment by Edman degradation led to the identification of V^{109} as *N*-terminal amino acid, indicating that both proteinases cleave at the same position within the CDR H3 loop of the antibody. Interestingly, this cleavage site (LFG^{108}↓V^{109}PIAR) is also found *in planta* (see Section 3.1). The *N*-terminus of the 30-kDa fragment produced by cathepsin L was determined by MS as T^{240}, located within the hinge region connecting the C_H1 and C_H2 domains. This cleavage site (KTH^{239}↓T^{240}CPPC) is identical to that previously described for papain [29].

Incubation of 2F5 with the serine proteinases chymotrypsin and subtilisin also led to rapid appearance of a 40-kDa fragment. Prolonged treatment with subtilisin yielded small amounts of a 30-kDa processing product,
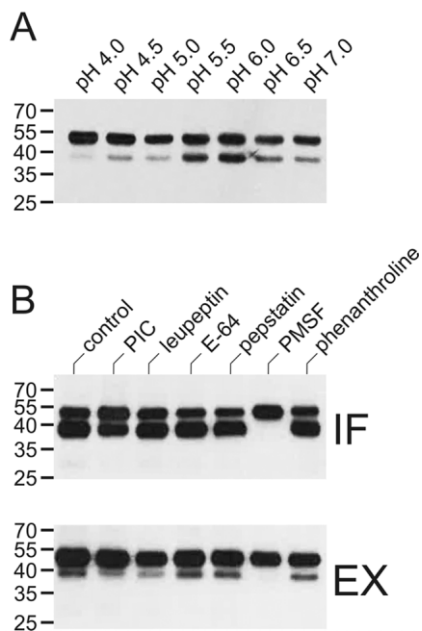

Figure 3. Effect of pH and proteinase inhibitors on in vitro processing of 2F5. (**A**) CHO-derived 2F5 (200 ng) was incubated with intercellular fluid (130 ng protein) for 16 h at the indicated pH and then analyzed by immunoblotting with antibodies to the heavy chain of human IgG. (**B**) CHO-derived 2F5 (200 ng) was incubated with intercellular fluid (IF; 650 ng protein) or total soluble leaf extracts (EX; 200 ng protein) for 16 h in the absence (control) or presence of the indicated proteinase inhibitors (10 μM: leupeptin, E-64, pepstatin; 2 mM: PMSF, phenanthroline) and then analyzed as above. PIC, proteinase inhibitor cocktail. The migration positions of selected molecular mass standards are indicated, with their respective masses expressed in kDa. The results shown are representative of at least two independent experiments.

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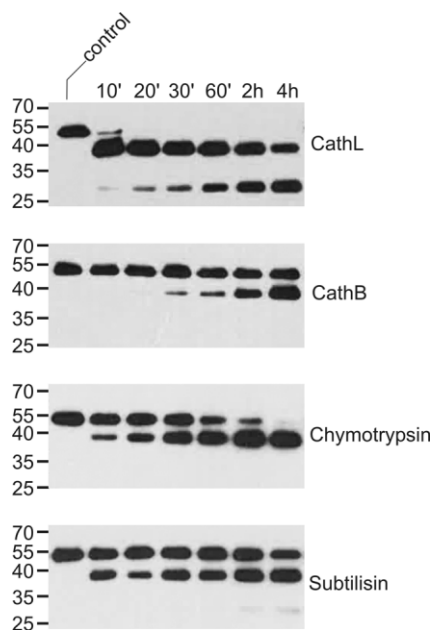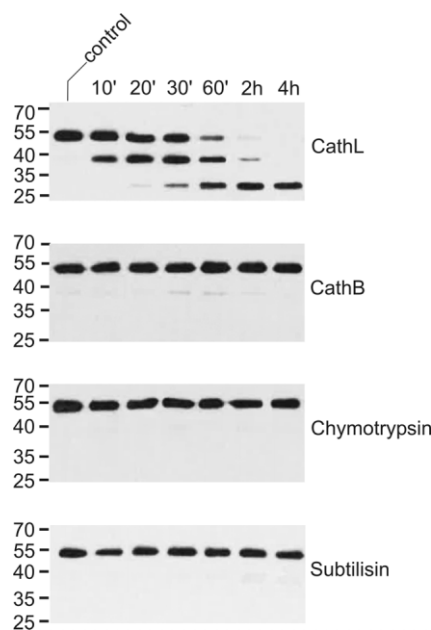visions
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Figure 4. Processing of 2F5 by serine and cysteine proteinases. CHO-derived 2F5 (200 ng) was incubated with selected proteinases (10 ng) at pH 5.5 for the indicated times and then analyzed by immunoblotting with antibodies to the heavy chain of human IgG. Untreated antibody was used as control. CathB, cathepsin B; CathL, cathepsin L. The migration positions of selected molecular mass standards are indicated, with their respective masses expressed in kDa. The results shown are representative of at least two independent experiments.

Figure 5. Processing of 2G12 by serine and cysteine proteinases. CHO-derived 2G12 (200 ng) was incubated with selected proteinases (10 ng) at pH 5.5 for the indicated times and then analyzed by immunoblotting with antibodies to the heavy chain of human IgG. Untreated antibody was used as control. CathB, cathepsin B; CathL, cathepsin L. The migration positions of selected molecular mass standards are indicated, with their respective masses expressed in kDa. The results shown are representative of at least two independent experiments.

while this was not observed for chymotrypsin (Fig. 4). The N-terminus of the 40-kDa fragment produced by subtilisin could not be identified unambiguously due to heterogeneity. However, the N-terminal sequence of the 40-kDa polypeptide generated by digestion with chymotrypsin was found to correspond to $G^{108}VPIA$, identical to the main cleavage site within the CDR H3 loop of 2F5 observed *in planta*.

In contrast to 2F5, 2G12 was hardly sensitive to treatment with cathepsin B, chymotrypsin, and subtilisin. The latter antibody also proved far more resistant to digestion with cathepsin L. However, the sequential appearance of the 40- and 30-kDa fragments still occurred, albeit much delayed as compared to 2F5 (Fig. 5).

A number of enzymes was found to display no or very little activity toward 2F5 when tested under apoplastic conditions. This includes the serine proteinase trypsin, the aspartic proteinase cathepsin D and the cysteine proteinase legumain (Supporting information, Fig. S3A). The same was observed when cathepsin D and legumain were used at pH 4.5, which is more favorable for their enzymatic activities (data not shown). However, prolonged incubation of 2F5 with high amounts of legumain resulted in the formation of a 30-kDa degradation product (Supporting information, Fig. S3B). The N-terminus of this fragment was identified by Edman degradation as K^{237}.

Interestingly, the same cleavage site ($SCD^{236}{\downarrow}K^{237}THTC$) was observed in the case of a mAb fragment isolated from tobacco leaves [12].

4 Discussion

Fragments of mAbs heterologously expressed in tobacco (*Nicotiana tabacum*) or *N. benthamiana* have been observed in a number of previous studies [11, 25, 30–32]. However, the molecular characterization of the cleavage products has been superficial so far [12, 33]. Although it is possible that some degradation products might have escaped detection in our studies, the presented data indicate that proteolytic fragmentation of 2F5 and PG9 occurs mainly by cleavage in the CDR H3 loop of the antibodies. This coincides with the site of 2G12 proteolysis when produced in transgenic maize, although the exact processing sites could not be identified [24]. Different findings have been reported for two other mAbs. In the case of the tumor-targeting antibody H10, MS-based peptide fingerprinting indicated that proteolysis took place in the C_H1–C_H2 hinge region [33]. The same was observed for the IgM-neutralizing antibody LO-BM2 expressed in tobacco BY-2 cells [12]. Interestingly, such a 30-kDa degradation product was observed for 2F5, but not for

PG9. This could be related to different efficiencies in heavy-light chain assembly, which could possibly expose the hinge region to proteolytic attack. Such a scenario would be in agreement with our observation that formation of the 30-kDa 2F5 fragment was most prominent when light-chain synthesis was reduced.

The presented in vitro experiments were all performed with CHO-derived mAbs as substrates, which differ from their plant-made counterparts with respect to their N-glycan structures. Since the single N-glycan attachment site is located in the Fc part and thus sterically remote from the cleavage regions, it is unlikely that differences in N-glycosylation would affect the proteinase sensitivity of the antibodies. This notion is supported by the observation that the proteolytic susceptibility of 2F5 produced in $N. benthamiana$ and CHO cells is comparable. Furthermore, 2G12 isolated from $N. benthamiana$ leaves was equally resistant to apoplastic proteinases as its CHO-derived counterpart (data not shown).

Although the antigen-binding sites of 2F5 and 2G12 have been grafted onto the same IgG1κ framework, the two antibodies differ markedly in their sensitivity to proteolysis in planta and in vitro. It is likely that this is at least partially due to the unusual architecture of 2G12, which features a swap between the variable domains of its two heavy-chain molecules, resulting in the formation of a third antigen-binding site at the newly created interface [34]. Thus, the domain-exchange possibly restricts access to the CDR H3 loop, the primary proteinase-sensitive region in the far more susceptible conventional mAbs 2F5 and PG9. Furthermore, 2G12 contains a particularly adaptable linker region between the V_H and C_H1 domains, which adopts a buried conformation as compared to a 2G12 variant with a canonical tertiary structure [35]. Hence, the relative inaccessibility of the CDR H3 loop and the V_H–C_H1 linker segment could account for the high resistance of 2G12 to proteolytic enzymes.

It should be also noted that 2F5 and PG9 are mAbs with very long CDR H3 loops. PG9 has a 28-residue CDR H3, which equals the longest in human antibody sequences known to date [36]. This structural element is only slightly shorter in the case of 2F5 (22 residues). In contrast, the CDR H3 loop of 2G12 is more compact with a length of 14 amino acids. Hence, the length of the CDR H3 loop correlates well with the sensitivity of these mAbs to proteolytic degradation in $N. benthamiana$. This notion is further supported by the positions of the primary cleavage sites with the CDR H3 loops of 2F5 and PG9, which are invariably located close to the loop tip and therefore sterically exposed.

It has been recognized already some time ago that proteolytic degradation in planta poses a serious constraint for the production of mAbs in tobacco and other $Nicotiana$ species, with this undesired turnover limiting both yield and quality of the protein of interest [37]. Previous studies have consistently reported acidic pH optima

for mAb-hydrolyzing activities in tissue extracts [10, 37]. This argues in favor of the involvement of proteolytic enzyme(s) accumulating in the intercellular fluid, in line with the apoplast being a proteinase-rich environment [5]. Support for this notion is also derived from the finding that fragments of the mAb Guy's 13 expressed in tobacco largely accumulate in the intercellular fluid [10]. However, the issue of the identity of the $Nicotiana$ proteinases accounting for mAb degradation is still unresolved. Interestingly, co-expression of tomato cystatin 9, an inhibitor of papain- and legumain-like cysteine proteinases, was reported to stabilize the murine mAb C5-1 in $N. benthamiana$. The same was observed for tomato cathepsin D inhibitor, which also targets serine proteinases [11]. These results are in good agreement with our findings that serine and cysteine proteinases are largely responsible for mAb degradation in $N. benthamiana$, thus providing important clues for future studies aiming at the identification of the detrimental host proteinases.

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