**Site targeted mutagenesis for stabilisation of a recombinant monoclonal antibody expressed in tobacco plants.**

**Verena K. Hehle, Matthew J. Paul, Victoria A. Robertsa, Craig J. van Dolleweerd\* and Julian K-C. Ma\***§

Molecular Immunology Unit, The Institute for Infection and Immunity, St George’s, University of London, Cranmer Terrace, London SW17 0RE, UK

a San Diego Supercomputer Center, University of California, San Diego, La Jolla, California 92093 USA

\* These Authors contributed equally

§Corresponding author:

Prof. Julian Ma

Tel: +44 208 725 5818

jma@sgul.ac.uk

Short title: Engineering stability in plant antibodies

**List of abbreviations:**

ELISA – enzyme linked immunosorbent assay

HRP – horseradish peroxidase

mAb – monoclonal antibody

MALDI-TOF - Matrix-assisted laser desorption/ionization – time of flight

Mr – relative molecular mass

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PVDF - Polyvinylidene fluoride

WT – wild type

**Abstract**

This study examined the degradation pattern of a murine IgG1κ monoclonal antibody expressed in and extracted from transformed *Nicotiana tabacum*. Gel electrophoresis of leaf extracts revealed a consistent pattern of recombinant immunoglobulin bands, including intact and full length antibody, as well as smaller antibody fragments. N-terminal sequencing revealed these smaller fragments to be proteolytic cleavage products, and identified a limited number of protease sensitive sites in the antibody light and heavy chain sequences. No strictly conserved target sequence was evident, although the peptide bonds that were susceptible to proteolysis were predominantly and consistently located within or near to the interdomain or solvent exposed regions in the antibody structure. Amino acids surrounding identified cleavage sites were mutated in an attempt to increase resistance. Different Guy’s 13 antibody heavy and light chain mutant combinations were expressed transiently in *Nicotiana tabacum* and demonstrated intensity shifts in fragmentation pattern resulting in alterations to the full length antibody to fragment ratio. The work strengthens the understanding of proteolytic cleavage of antibodies expressed in plants and presents a novel approach to stabilise full length antibody by site directed mutagenesis.

**Key words**

Degradation; proteolysis; antibody engineering; Nicotiana.

**Introduction**

Plant biotechnology has become widely used for recombinant pharmaceutical protein expression (molecular pharming) since proof of concept over 25 years ago ([1](#_ENREF_1)). The field has advanced swiftly with the first FDA approved drug in 2012; ELELYSO™ an enzyme produced in carrot cell suspension culture for treatment of Gaucher disease ([2](#_ENREF_2)) and a number of products in clinical trials ([3](#_ENREF_3), [4](#_ENREF_4)). Recently, a plant produced, experimental monoclonal antibody cocktail ZMapp was provided for compassionate use to treat humans infected with the Ebola virus.

Monoclonal antibodies have been the focus of attention for many groups, but a number of difficulties still need to be addressed, in order to maximise antibody yield from plant manufacturing systems. In particular, the quality of plant-derived IgG monoclonal antibodies can be dramatically affected by unintended proteolysis, and this has been observed in both stable transgenic plant and transient expression systems ([5](#_ENREF_5)). Relatively little is known about the specificity of antibody degradation in plants. Western blots of recombinant mAbs expressed in plants invariably show a number of immunoreactive bands, in addition to the putative full-length antibody ([5-7](#_ENREF_5)). Previously, these bands have been explained as incomplete or partial assembly intermediates of the immunoglobulin heavy and light chains ([8](#_ENREF_8), [9](#_ENREF_9)), based on previously identified assembly intermediates from murine lymphoid and malignant plasma cells ([10](#_ENREF_10)). However, more recently, it has been demonstrated that many of the detected fragments are actually degradation products that detract significantly from the productivity of the expression system ([5](#_ENREF_5), [7](#_ENREF_7)). A variety of attempts have been made to overcome this problem, for example by inclusion of protease inhibitors in extraction buffer or by co-expression of protease inhibitors ([11-13](#_ENREF_11)). Protease activity *in vivo* may also be inhibited by gene silencing strategies ([14](#_ENREF_14)). Methods for improving transcription and translation levels have been investigated ([15](#_ENREF_15), [16](#_ENREF_16)), as well as methods for enhancing the stability of the product protein ([17](#_ENREF_17)), by targeting the antibodies to specific subcellular compartments ([18](#_ENREF_18), [19](#_ENREF_19)), by glycan engineering ([20](#_ENREF_20)) or by fusing other proteins to the antibody ([21](#_ENREF_21), [22](#_ENREF_22)).

However, none of these approaches have been able to significantly reduce proteolysis. It has been shown that recombinant antibodies, depending on their primary sequence, structural characteristics and subcellular localisation, are likely to contain amino acid sequences that are targeted by peptidases in plant cells ([5](#_ENREF_5), [7](#_ENREF_7), [23](#_ENREF_23)) particularly as these heterologous proteins have never evolved in the context of the host protease environment. It was recently shown that there are only a limited number of plant proteolytic cleavage events in human immunoglobulin light and heavy chains, and that these were usually focused at exposed sites of inter-domain regions of each immunoglobulin chain ([5](#_ENREF_5)).

Endopeptidases show a variety of sequence specificities surrounding the cleavage site. Some cleave polypeptides at specific motifs which, in turn, are characteristic of the peptidase, while others show a very broad recognition spectrum ([24](#_ENREF_24)). For example, trypsin cleaves exclusively after Lys or Arg residues (at P1) ([25](#_ENREF_25)). Proline usually blocks this action when found in position P1', carboxyterminal of the scissile bond. In contrast, the plant proteases pepsin and papain have fairly broad specificity ([24](#_ENREF_24)).

Amino acid mutations that confer resistance to proteolysis might have a measurable effect on the antibody fragmentation pattern. Expression of antibodies incorporating these mutations might therefore result in simplified antibody purification from plants and improved yields of fully assembled, functional mAbs. In the present study an approach consisting of engineering protease resistance into antibody sequences by targeting susceptible cleavage sites was explored. Amino acids surrounding the identified cleavage sites were modified, with the aim of preventing proteolytic degradation of plant expressed mAb Guy’s 13. It was demonstrated that mutations of residues immediately proximal to identified cleavage sites modulate, but not completely eliminate proteolytic degradation of monoclonal antibody.

**Material and Methods**

**Transgenic plant material**

Transgenic *Nicotiana tabacum (N. tabacum* var. Petit Havana)lines homozygous for both the 1 heavy and  light chain genes of the murine IgG1 mAb Guy’s 13 ([26](#_ENREF_26)) were used.

## Mutagenesis of mAb Guy’s 13 heavy and light chain

The 1 heavy and kappa light chain genes of mAb Guy’s 13 had previously been cloned between the *Xho*I and *Eco*RI sites of pL32 and clones, designated 1#3 and 4.1 were used in this study ([26](#_ENREF_26)). Using the QuikChange® (#200518, Stratagene, UK) mutagenesis protocol according to manufacturer’s instructions, oligonucleotide primers (see supplementary data) were used to introduce site directed mutations. Overlapping regions of the heavy or light chain were amplified. PCR products were annealed via their common overlap and amplified in a second PCR reaction, purified and ligated into the plant expression vector pL32. After transformation of *E.coli* XL10-Gold (Stratagene) individual colonies were screened by digestion with the appropriate restriction enzymes (supplementary table) for each individual mutant. Putative mutants identified by this analytical restriction enzyme digest were confirmed by sequencing (Beckman Coulter Genomics) prior to transformation of *A. tumefaciens* EHA105.

## Transient expression in *N. tabacum* by agroinfiltration

For transient expression, the heavy and light chain genes of mAb Guy’s 13 were expressed from a plant transformation vector (pL32) ([26](#_ENREF_26)). Wild type *N. tabacum* plants were cultivated for 10-11 weeks from seed. Recombinant *Agrobacterium tumefaciens* cultures EHA105 harbouring the light and heavy chains of Guy’s 13 were grown overnight at 28°C, with shaking at 250 rpm, in Luria Bertani medium supplemented with spectinomycin (200 g/ml) and rifampicin (100 g/ml). Cultures were centrifuged for 5 min at 8000x*g* and for co-infiltration of heavy and light chains, aliquots of resuspended cell pellets (in MS media) were combined to give a total volume of 1.5 ml. The bacterial solution was injected directly using a syringe, pressed firmly against the abaxial surface of a leaf ([27](#_ENREF_27)). The plants were left to recover under standard growth conditions (temperature 25oC, 16/8 hrs light/dark cycle) for 5-7 days before leaves were harvested for analysis of the recombinant protein.

## Extraction of mAbs from transgenic and transiently expressed agroinfiltrated tobacco plants

Tissue from mature leaves of transgenic tobacco plants expressing mAb Guy’s 13 were homogenized with three volumes of PBS at room temperature. After two cycles of 20 seconds of homogenisation using a Waring blender, the plant extract was centrifuged at 17,000 *g*, for 30 min at 10°C. The supernatant was passed through Whatman #3 filter paper, and immediately placed on ice. The pH of the filtered plant juice was adjusted to pH 7.5-8.0 with 1 M NaOHand incubated for at least 30 min on ice, followed by re-centrifugation at 40,000 *g* for 20 min at 10°C. The supernatant was filtered through a 0.22 m Millex GP Filter (Millipore, Consett, Co Durham, UK) and stored at -20°C until required. For antibody purification from agroinfiltrated *N. tabacum* plants, infiltrated leaves were sampled and homogenised for 5 min at 29 oscillation/s using a Mixer Mill MM 400 (Retsch). Samples were centrifuged at 17,000 g for 10 min at 10°C and the supernatant stored at -20°C until required.

For affinity purification, Protein G-Sepharose® 4B resin (Sigma) and protein A-agarose (Sigma) (1:1 mix) were packed into a glass chromatography column (BioRad) to give a final bed volume of ~1 ml. Filtered supernatant was applied at a flow rate of 0.5-1 ml/min. The column was washed with ≥20 column volumes of PBS and elution was with 0.1 M glycine (pH 2.5), in 1 ml fractions. Fractions were neutralized with 1 M Tris base (pH unadjusted).

To concentrate the samples, the pooled fractions were transferred to 50 ml Falcon tubes and freeze dried under vacuum overnight. Lyophilized samples were resuspended in 200 l dH2O and dialysed overnight against PBS.

**Western blotting**

Protein transfer was performed for 90 min onto a Hybond nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 0.4 mA/cm2 and 50 V using a semi-dry blotting device (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The membrane was incubated with 5% (w/v) non-fat milk powder (MARVEL original, Dried Skimmed Milk) in TBS for 30 min to block non-specific binding sites. Detection of proteins was with goat anti-murine IgG, Fcsubclass1 antiserum (Jackson ImmunoResearch, Suffolk, UK, 115-035-205) and goat anti-murine IgG, kappa light chain specific antisera (Jackson ImmunoResearch, 115-035-174) for 1 hr at room temperature. The membrane was washed five times with 0.1% Tween-20 in TBS (5 min per wash), then developed using the ECL Plus western blotting detection system (GE Healthcare, Little Chalfont, UK).

**N-terminal sequencing**

Purified mAb Guy’s 13 samples were separated by SDS-PAGE on 4-15% gels (Bio-Rad), blotted on Polyvinylidene fluoride (PVDF) membrane and stained with Coomassie suspension G250. The N-terminal sequencing of mAb degradation fragments was performed by Mike Weldon (University of Cambridge) on a Procise® Protein Sequencing System (Applied Biosystems, Foster City, CA).

### Densitometry

Band density from western blots was measured by densitometry (GeneTools, Syngene, Cambridge, UK). Individual infiltrations were analysed using student’s t-test (p <0.05) and the raw values for the fully assembled antibody compared to the raw values of all other bands present in the samples.

**ELISA**

For quantification of expressed mutant antibodies or functional antigen binding ELISA, 96 well microtitre plates (NUNC MaxiSorp™, Thermo Scientific) were coated for 2 hrs at 37°C with capture antibody- anti-murine kappa light chain (Jackson ImmunoResearch, 115-035-174) at 50 l/well or with recombinant *E. coli*-derived version of SAI/II in PBS buffer normally at 5g/ml, respectively. The plates were washed once with distilled H2O before blocking with 200 lwellPBS buffer containing 5% (w/v) non-fat milk powder for 2 hrs at 37°C, or overnight at 4°C. Samples were loaded at 50l/well, titrated accordingly and incubated for 2 hrs at 37°C. Plates were then washed three times with distilled H2O containing 0.1% Tween 20 (H2O/T20). For detection, secondary antibody (anti-murine gamma chain antiserum, Sigma, UK or anti-murine kappa chain antiserum Sigma, UK) labelled with horseradish peroxidase (HRP) was added for 2 hrs at 37°C. Finally the plates were washed three times with H2O/T20 and developed with TMB solution, 50l/well. The reaction was stopped by adding 50 l of 2 M H2SO4 and the plates were read using a Sunrise plate reader (Tecan) at 450 nm. To determine antibody concentrations, a standard amount of quantified, commercially available antibody was also used, and titrations fitted with a sigmoidal dose-response curve to obtain the EC50 (Graphpad Prism™, Graphpad Software).

**Results**

**Guy’s 13 fragmentation pattern**

The murine monoclonal antibody (mAb) IgG1 Guy’s 13 was expressed by stable nuclear transformation or by transient expression in *Nicotiana tabacum*. Leaf extracts were separated by SDS-PAGE and the presence of intact antibody as well as antibody fragments was detected by western blotting (Figure 1). The results indicate close similarity in the antibody fragments regardless of which expression system was used. Detection with anti-Fc antiserum (panel A) identified three major bands at Mr ~180K (band \*, presumed to represent fully assembled antibody), Mr ~150K (band a) and Mr ~100K (band b) in both of the antibody expressing plant samples, but not the wild-type (WT) non transgenic *N. tabacum* leaf extract (lane 1). A similar result was obtained using an anti-kappa chain antiserum (panel B), although in this case, more immunoreactive bands were observed. There were subtle differences between the relative intensities of individual bands, for example bands \* and a are more intense in the transiently expressed sample (lane 6), but bands b, c and d are more intense in the transgenic expression (lane 5). Some minor bands were observed in one expression system but not the other (eg bands e and f). Again, the WT control *N. tabacum* demonstrated no immunoreactivity.

**Identification of N-terminal cleavage sites in mAb Guy’s 13**

Affinity purified Guy’s 13 fragments from a batch of transiently expressed mAb were separated by non-reducing SDS-PAGE, transferred onto PVDF membrane and stained with Coomassie G250 (Figure 2). The most prominent bands - labelled a, b, c, d and f, were present as before and Edman degradation was applied to these bands to analyse the amino acid sequences of their N-termini. For bands a, b and c, N-terminal sequences were returned corresponding to the correctly processed N-termini of both the light and heavy chains. For both fragments d and f, N-terminal sequences were identified from within both the light and heavy chains. A single light chain N-terminus amino acid sequence was identified (EIKR), which resides within the junction between VL and CL domains. Similarly only a single heavy chain amino acid sequence was identified (AKTT) which corresponds to the junction between VH and CH1 domains.

Additionally, an N-terminal sequence was identified for a band detected following SDS-PAGE under reducing conditions (data not shown) at Mr ~17K starting with the amino acids RFSG which is found within the VL region of Guy’s 13, near the boundary with CL.

### Mutagenesis of heavy or light chains of mAb Guy’s 13

Having identified a very limited number of protease susceptible sites in the heavy and light chains of mAb Guy’s 13, a series of mutations were designed up and downstream of the P1 and P1’ amino acids (Figure 3) and expressed. Several mutagenesis approaches were considered. 1) Conservative substitution using amino acids with the same physicochemical properties; 2) Non-conservative substitution using amino acids with divergent physicochemical properties; and 3) Structurally conservative amino acid changes aimed at preserving the three-dimensional structure of the antibody. In the first two cases, amino acid substitutions were made using the Kabat database ([28](#_ENREF_28)) to assess the range of natural variants at these positions. If no possible natural variant was available in the Kabat database, changes were made using amino acids which were physicochemically most similar/or divergent to the original Guy’s 13 residue. In the third approach, in silico modelling, performed with the molecular graphics programs Insight (Accelrys, Inc.) and RasMol ([29](#_ENREF_29)), was used to choose substitute amino acid residues, taking into account side chain interactions so as not to interfere with the tertiary structure of the antibody.

Four mutants were produced for the identified heavy chain sequence AKTT (Figure 3), two representing conservative substitutions (HC1 and HC3), and two representing non-conservative substitutions (HC2 and HC4). Eight light chain mutants were also produced, two representing conservative substitutions (LC1 and LC3), two representing non-conservative substitutions (LC2 and LC4) and four representing structurally conservative substitutions (LC5-LC8).

**Expression and characterisation of IgG mAb with mutated heavy and light chains**

Various combinations of heavy and light chains were transiently expressed in *N. tabacum.* Extracts from infiltrated leaves were collected and analysed by non-reducing SDS-PAGE followed by western blotting with anti-murine kappa chain antiserum. Figure 4 illustrates the results from one experiment that was representative of at least 3 repeat experiments for each mutant antibody construct. In some cases, for example mutants LC5 and HC1, no significant differences were observed in mAb expression when the mutated chains were co-expressed with the original non-mutated corresponding immunoglobulin chain. This was confirmed by densitometry, which estimated the percentage contribution of the full length antibody band (denoted with a \*) to be 16.8 and 26% respectively, compared with 23.5% for co-expressed non-mutated heavy and light chains. Similar results were obtained with mutants HC3 and LC6 (not shown)

For both HC4 (co-expressed with non-mutated light chain), and LC3 (co-expressed with non-mutated heavy chain), there was a marked reduction in full length mAb expression. For HC4 no assembled mAb was detected, whereas for LC3, full length mAb represented only 5.4% of the total immunodetected bands. A similar reduction in full length mAb yield was also observed for mutants LC2 and LC4 (not shown).

In three cases, an apparent increase in full length mAb accumulations was observed, even though there did not appear to be a significant overall increase in light and heavy chain expression. These were HC2 (co-expressed with non-mutated light chain), LC1 and LC8 (co-expressed with non-mutated heavy chain) and to some extent, LC7 (not shown). In the case of HC/LC1, all the bands identified in the non-mutated antibody were present, but the proportion of full length antibody was enhanced. For HC2/LC and HC/LC8 combinations, bands f, and c, d, e and f were significantly diminished respectively. For HC2, LC1 and LC8, the full length mAb was the most prominent band on the western blot, representing between 36.5-43.8% of the total immunodetected bands. No relationship was discerned between the mutagenesis substitution strategy used and effects on antibody stability.

The assembled HC2 mutant IgG had an increased mobility in antibody bands on Western blot membranes, that was not seen with other mutants (Figure 4). Interestingly this mobility shift affected all the major bands detected in the Western blot. Under reducing conditions, no difference was observed between the relative molecular masses of HC2, non-mutated heavy chain or other mutants (data not shown). MALDI-TOF spectra of HC2 and HC were also comparable (data not shown), indicating that the shift in migration observed for HC2 mutant antibodies was not due to a truncated heavy chain.

**Co-expression of mutated heavy and light chains of mAb Guy’s 13**

IgG yield was also assessed for combinations of HC mutants with LC mutants (Figure 5). Light chain mutants that had had a positive effect (LC1, LC7 and LC8) or no effect (LC5 and LC6) were co-expressed with HC1 (no effect) or HC2 (positive effect). None of the light chain mutants when co-expressed with HC1 resulted in greater yield of intact IgG (Panel A). This was irrespective of whether or not the light chain mutant itself had individually resulted in an improvement in IgG yield (Figure 4). When HC2 mutant heavy chain was co-expressed with LC5 or LC6, there was no enhanced IgG yield (Figure 5 panel B), even though HC2 with LC (non-mutated) had previously resulted in improvement (Figure 4). The combination of HC2 with LC7 (both associated individually with improved IgG yield) did not result in significant improvement.

However, two combinations of HC and LC mutants did result in significant improvement of intact antibody yield. These were HC2 with LC1 and HC2 with LC8 (Figure 6). In both cases, both the heavy chain mutant (HC2) and the light chain mutants were associated individually with higher yield of intact IgG (Figure 4). For the HC2/LC1 combination, both bands d and f were eliminated.

Replicate experiments were performed with nine (HC2 + LC1) and six (HC2 + LC8) independent infiltrations, and the percentage of intact IgG was assessed by densitometry (Figure 7, panels A and B respectively). In each case, the non-mutated HC + LC control was infiltrated into the same plant. The data resulting from densitometry analysis confirmed the relative abundance of the fully assembled antibody to its associated fragments. All infiltrated mutant combinations (HC2 + LC1 and HC2 + LC8) showed significant differences in the percentage of fully assembled IgG, compared to the IgG in the control infiltration (HC + LC). In all cases, the percentage of fully assembled antibody was approximately doubled by introducing the mutations.

To confirm that Guy’s 13 light and heavy chain mutants retained binding activity to the relevant antigen (streptococcal antigen I/II), a functional ELISA was performed (Figure 7, panel C). Here, the positive control was an extract from *N. tabacum* infiltrated with Guy’s 13 HC + LC. All antibody extracts were applied in triplicate at the same concentration, as determined previously by quantitation ELISA (data not shown). Detection of antibody bound to SAI/II was with an HRP-conjugated anti-murine kappa chain antibody. All mutants demonstrated a clear positive signal indicating that they are assembled and bind to SA I/II. EC50 dilutions were calculated for each antibody and are shown in Figure 7, panel C. There were no significant differences between the control HC + LC and any of the mutants (students t-test, p < 0.05).

**Discussion**

The development and use of recombinant monoclonal antibodies (mAbs) represents one of the leading research areas for therapeutics diagnostics and vaccine development. Alternative expression platforms such as plants are being explored to address issues of cost and scalability, especially for applications targeted to resource-poor regions. However, fully assembled antibody in plants is usually accompanied by additional immunoglobulin species of lower molecular weight, which are predominately generated by enzymatic action of plant proteases ([6](#_ENREF_6), [7](#_ENREF_7), [23](#_ENREF_23)). This consistent problem reduces yields, complicates downstream processing and increases the production costs ([30](#_ENREF_30)).

Plant proteases are often pivotal to maintaining metabolic functions, so in this study, we developed an approach of modification of specific proteolytic target sequences in a monoclonal antibody to improve expression in plants. This could be achieved without affecting antibody assembly and function. Our previous findings with other monoclonal antibodies had indicated the presence in antibodies, of a limited number of conserved cleavage sites that are located in interdomain and solvent exposed regions of the light and heavy chains ([5](#_ENREF_5)). These findings were confirmed here, using a murine IgG mAb, by N-terminal sequencing of antibody fragments present in crude plant extract. In particular, an important cleavage site (EIKR) was identified in the light chain at the junction of VL and CL, and similarly a major site (AKTT) was found in the heavy chain at the junction of VH and CH1. Whether EIKR and AKTT actually represents the exact N terminus of a newly cleaved polypeptide remains to be determined, as it is possible that primary cleavage upstream of these sites might be followed by further trimming by other proteases. Other proteolytic sites towards the C-terminus of light and heavy chains are likely and indeed predicted from our results, but despite extensive analysis, no other new cleavage sites have been identified in the variable domains or in the constant or CH1 domains of light and heavy chains respectively.

Previous studies have demonstrated the principle of antibody engineering to enhance stability. For example, the stability of human IgA1 against bacterial proteases was enhanced through engineering of a hybrid hinge region comprising half of human IgA1 and human IgA2 hinge region ([31](#_ENREF_31)). Peters et al. demonstrated that mutations introduced into the CH1 domain of IgG4 increased antibody thermal stability by mimicking the interchain disulphide bond arrangements observed in IgG1 ([32](#_ENREF_32)). In our study, a range of mutations targeted at the EIKR and AKTT sites in the antibody light and heavy chains were examined. Sequences were identified that either abolished or significantly diminished full length antibody yield, had no apparent effect, or that enhanced full length antibody yield. Although different approaches were used to design the mutations, no consistent relationship between mutation strategy and antibody yield was evident, so at this stage, a mechanism of action can only be speculated on. One possibility is that the change to a more hydrophobic amino acid in position 113 (Ser113Val) and a basic amino acid in position 114 (Ala114His) of Guy’s 13 heavy chain (mutant HC2) may alter the accessibility of the site, as both residues were found in a solvent exposed, interdomain region. Proteins typically fold in such a way to minimize solvent exposure of the hydrophobic amino acids while exposing the hydrophilic residues. Appropriate presentation of a potential cleavage site in an exposed and unstructured region (such as a solvent exposed loop) would be a minimal requirement for hydrolysis. The replacement of polar amino acids for hydrophobic amino acids (as done in mutant HC2) might prevent or slow down access to the cleavage site.

Of interest was the finding that an alteration in the light chain, eg LC8, could result in the loss of multiple bands including apparently, those linked to heavy chain degradation. This led us to consider the likelihood that antibodies expressed in plants are cleaved by different proteases in a sequential manner. Thus proteolysis at an initial site on the light chain, might allow subsequent protease access to additional amino acid sequences, previously buried within the antibody structure including on the heavy chain. Similar findings have been reported, for example in the caseof sphingosin kinase-1 (SK1) cleavage by cathepsin B. Site-directed mutagenesis of an initial target site, not only abrogated a Mr 30K degradation band and stabilised intact SK1, it also reduced the appearance of an additional Mr 18K fragment ([33](#_ENREF_33)).

An unexpected finding was that the expression of HC2 mutant combinations were associated with a consistent shift in apparent molecular mass on polyacrylamide gel electrophoresis, relative to the control (HC + LC) and molecular weight marker. The possibility of a truncated HC2 mutant, cleaved by proteases, was excluded by MALDI-TOF analysis (data not shown). A comparison of the calculated pIs for the wild type HC (pI = 6.55) and the HC2 (pI = 6.59) revealed only a minor effect on the overall charge of the HC2 mutant. Changes in hydrophobicity of the HC2 mutant may explain the phenomenon of gel shifting, as Val113 is more hydrophobic than the substituted Ser113, whereas His114,isa more basic, cationic amino acid in character than Ala114 ([34](#_ENREF_34)).

The next stage of this work will be to extend the analysis of protease cleavage sites to other similar antibodies, to identify common and consistent target peptide motifs. It should ultimately be possible to perform more extensive screening of mutation strategies and to assess combination approaches that provide maximum IgG protection. The initial findings reported here for a monoclonal antibody could, of course, be extended to other recombinant proteins expressed in plants. In addition, the targeted mutation approach could be used to complement strategies that involve protease inhibition ([23](#_ENREF_23)) and or plant protease knockout ([7](#_ENREF_7)) that are being adopted elsewhere, alongside multiple other approaches to maximise recombinant protein accumulation in plant systems.

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**Legends to figures:**

**Figure 1: Western blot analysis of mAb Guy’s 13 expressed in *N. tabacum*.**

Samples from mAb expressed transgenically or transiently in *N. tabacum* were separated on a non-reducing 4-15% SDS-PAGE gel and proteins were blotted onto nitrocellulose membrane. For detection, anti-Fc (panel A, lanes 1 - 3) and anti-kappa (panel B, lanes 4 - 6) antisera were used. Lanes 1 and 4 - non-transgenic *N. tabacum* (WT); lanes 2 and 5 - crude leaf extract of transgenic *N. tabacum* plants expressing Guy’s 13 (transgenic); lanes 3 and 6 – crude leaf extract from transiently expressed mAb Guy’s 13 (transient). Asterisk represents fully assembled antibody. Lower case letters (a to f) represent antibody fragments.

**Figure 2: N-terminal sequencing results of non-reduced mAb Guy’s 13 fragments.**

N-terminal sequencing of protein A/G purified mAb Guy’s 13 blotted onto PVDF membrane and stained with Coomassie G250. Arrows indicate position of N-terminal sequence within heavy and light chain amino acid sequence. Upper case letters indicate sequence returned from N-terminal sequencing by Edman degradation. Lower case letters indicate the major antibody fragments.

**Figure 3: Mutants of Guy’s 13 heavy and light chain with substitutions proximal to the cleavage sites ‘AKTTP’ and ‘EIKRA’.**

The wild type Guy’s 13 heavy and light chain sequence is shown in bold with corresponding Kabat amino acid numbering. The location of the protease cleavage site is indicated by the arrow and the positions amino terminal (P1 to P6) and carboxyl terminal (P1’ to P6’) to the scissile bond are shown at the top of the table.

The amino acid sequences used in mutant heavy and light chains are shown below, with amino acid substitutions shown (unshaded). Grey shading represents an unchanged amino acid residue.

**Figure 4: Transient expression of Guy’s 13 heavy and light chain mutants in *N. tabacum*.**

Non-reducing Western blot of leaf extracts from transiently-expressed Guy’s 13 heavy chain mutants (HC1, HC2, and HC4) expressed together with non-mutated Guy’s 13 light chain (LC) and light chain mutants (LC1, LC3, LC5, and LC8) together with non-mutated Guy’s 13 heavy chain (HC). Detection was with HRP-conjugated anti-murine kappa chain antiserum. Control was non-mutated Guy’s 13 heavy and light chains (HC + LC). The asterisk indicates the fully assembled IgG; lower case letters (a to f) indicate the major antibody fragments.

**Figure 5: Transient co-expression of Guy’s 13 heavy and light chain mutants in *N. tabacum*.**

Non-reducing Western blot of leaf extracts from transiently-expressed Guy’s 13 heavy chain mutants A) HC1 with Guy’s 13 light chain mutants (LC1, LC5, LC6, LC7 and LC8); or B) HC2 with Guy’s 13 light chain mutants (LC5, LC6 and LC7). Detection was with HRP-conjugated anti-murine kappa chain antiserum. Control was non-mutated Guy’s 13 heavy and light chains (HC + LC). The asterisk indicates the fully assembled IgG; lower case letters (a to f) indicate the major antibody fragments.

**Figure 6: Transient co-expression of Guy’s 13 heavy and light chain mutants in *N. tabacum*.**

Non-reducing Western blots of leaf extracts from infiltrated Guy’s 13 heavy chain mutant (HC2) co-expressed with Guy’s 13 light chain mutants (LC1 or LC8). Detection was with HRP-conjugated anti-murine kappa chain antiserum. Control was non-mutated Guy’s 13 heavy and light chains (HC + LC). The asterisk indicates the fully assembled IgG.

**Figure 7: Panels A and B: Densitometric analysis of antibody assembly from Western blots.**

The percentage of fully assembled antibody was measured by densitometry of nine biological repeats for HC2 +LC1 and HC2 +LC8. Data is mean­+sd. Independent infiltrations from *Nicotiana tabacum* of non-mutated Guy’s 13 control (HC + LC) was compared by student’s t-test (p<0.05) to infiltrated mutants HC2 + LC1 (panel A) and HC2 + LC8 (panel B) (\* =p<0.05; \*\*=p<0.005).

Panel C: Antigen binding activity for Guy’s 13 mutants. Equivalent amounts of each antibody preparation were serially diluted on an ELISA plate coated with SAI/II. Bound antibody was detected with HRP-conjugated anti-murine kappa chain reagent. Data is mean+sd. Absorbance values were plotted against dilutions. The titrations were fitting using a sigmoidal dose response curve (GraphPad Prism), and EC50 values calculated.