Antibody proteolysis: a common picture emerging from plants

We have recently characterized the degradation profiles of 2 human IgG1 monoclonal antibodies, the tumor-targeting mAb H10 and the anti-HIV mAb 2G12. Both mAbs were produced in plants either as stable transgenics or using a transient expression system based on leaf agroinfiltration. The purified antibodies were separated by 1DE and protein bands were characterized by N-terminal sequencing. The proteolytic cleavage sites identified in the heavy chain (HC) of both antibodies were localized in 3 inter-domain regions, suggesting that the number of proteolytic cleavage events taking place in plants is limited. One of the cleavage sites, close to the hinge region, was common to both antibodies.

In this work we report the characterization of mAb H10 degradation profile in plants by using 2-dimensional gel electrophoresis (2-DE) analysis, a technique that allowed us to separate protein species with higher resolution. By subsequent protein spots analysis we identified an additional cleavage site in the VH-CH1 inter-domain region of the HC. Interestingly, other cleavage sites in this region had been previously reported in the literature, supporting the hypothesis that different antibodies produced in plants share common degradation events, which appear to specifically occur in solvent accessible regions at domain interfaces.

Plants are ideal hosts for the production of heterologous proteins. With respect to traditional expression systems, based on bacterial and mammalian cells, plants offer several advantages, including reduced risk of contamination by human pathogens and low production costs. A major obstacle hampering the production of recombinant proteins in plants is represented by degradation phenomena, which may take place either in the cell or during downstream processes and result in a dramatic reduction of the final yield of intact heterologous proteins. Moreover, both the quality and yield of the recombinant protein may be significantly influenced by the intrinsic stability of polypeptide chains expressed in heterologous cell environment. Hundreds of genes encoding enzymes involved in proteolytic pathways have been identified in plants; as an example, about 800 genes directly or indirectly involved in the hydrolysis of peptide bonds are present in Arabidopsis thaliana. In the case of antibodies, unintended proteolysis driven by this complex peptidase repertoire can affect the final yield of intact IgG, and may lead to almost complete degradation. The presence of antibody fragments in plants has been ascribed to the following 3 phenomena: i) partial assembly intermediates; ii) extracellular peptidase activity after secretion; iii) activity of peptidases released during sample homogenization. Additionally, fragments resulting from a first “opening cleavage” might be further processed into smaller fragments by plant proteases. Recent studies have been focused on the identification of cleavage fragments resulting from in planta proteolysis by either mass spectrometry (MS) or N-terminal sequencing by Edman degradation. Mass analysis allowed 2 major degradation products to be identified, compatible with a cleavage presumably occurring close to the heavy chain (HC) hinge region of the H10 antibody. Edman degradation analysis led to the identification of a major cleavage site, which was consistent with the results obtained by MS analysis.

Keywords: 2DE, Agrobacterium tumefaciens, agroinfiltration, immunoglobulin, plant proteolysis, molecular farming, N-terminal sequencing, plant proteases

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Submitted: 05/13/2015
Revised: 06/24/2015
Accepted: 06/24/2015
http://dx.doi.org/10.1080/21655979.2015.1067740

Addendum to: Hehle VK, Lombardi R, van Dolleweerd CJ, Paul MJ, Di Micco P, Morea V, Benvenuto E, Donini M, Ma JK-C. Site-specific proteolytic degradation of IgG monoclonal antibodies expressed in tobacco plants. Plant Biotechnol J 2015; 13:235–45.
identification of the N-terminal sequence of a fragment of the chimeric rat/human Lo-BM2 antibody localized in the hinge region of the HC. In a recent study, Hehle and colleagues characterized by N-terminal sequencing the degradation profile of 2 human IgG1 monoclonal antibodies (mAbs) named 2G12 and H10 produced in tobacco plants. A limited number of proteolytic cleavage sites were identified in both the HC and light chain (LC) of the 2 mAbs, all of which are located within inter-domain regions.

Here we report the study of the degradation profile of the plant produced tumor-targeting mAb H10 determined by reducing 2-dimensional gel electrophoresis (2-DE) analysis. The mAb H10 was produced in Nicotiana benthamiana (N. benthamiana) plants by using a transient expression system based on agroinfiltration and subsequently purified by protein A affinity chromatography, as previously reported. For 2-DE analysis, solubilized protein samples were supplemented with 350 μL of isoelectrofocusing (IEF) rehydration buffer and incubated with IPG-strips 3-11NL/18 cm (GE Healthcare, Uppsala, Sweden) O/N at room temperature essentially as described by Di Carli and colleagues. Second dimension was run on 12.5 % (w/v) polyacrylamide gels using an Ettan DALT 12 unit (GE Healthcare, San Francisco, CA, USA) and gels were stained by Coomassie Blue as previously described. As shown in Figure 1A, we focused our attention on protein spots of about 15–25 kDa with an experimental isoelectric point (pI) range of 3–7. In previous studies we demonstrated that the HC is specifically cleaved in plants yielding protein fragments of about 15 kDa and 25 kDa on reducing gel electrophoresis. For this reason we expected that higher MW spots (~50 kDa) corresponded to the complete HC and the selected spots could represent HC-derived fragments. Spots 1–6 have a molecular weight of ~25 kDa, and are distributed within the pI range 4–7. In the pI range 3–4, 4 major spots are visible: spots 7 and 8, whose molecular weight is ~23 kDa and ~18 kDa, respectively; spots 9 and 10, whose molecular weight is ~20 kDa. To identify the molecular species associated with each spot, Western blot analysis was performed using anti-LC and anti-HC specific antibodies. Briefly, proteins were separated by 2-DE as reported above, blotted on a PVDF membrane (Millipore, Bedford MA) and incubated with either anti-human γ chain (8419; Sigma Aldrich) or anti-human λ chain (A5175; Sigma Aldrich) horseradish peroxidase labeled antibodies for 1h at room temperature in 2% (w/v) non-fat milk in PBS. Detection was performed using ECL Plus Western blotting reagent (GE; Healthcare). In the anti-LC Western blot analysis (Fig. 1B) major signals at 25 kDa corresponded to spots 1 to 6 observed in the Coomassie stained gel (spot 5 representing the most abundant one). Their molecular weight (~25 kDa) is in agreement with the presence of LC confirming previous results that showed no appreciable degradation of LC in plants and their different pI values are probably related to different post-translational modifications. Only a faint spot with lower molecular weight and acidic pI was observed, probably corresponding to spot 10 in Figure 1A.

Based on these results spots 7 and 10 were selected for N-terminal sequencing analysis. This involves a series of chemical
reactions to derivatize and remove one amino acid at the time from the N-terminal of purified peptides, enabling the sequential identification of N-terminal residues. N-terminal protein analysis was performed by Dr. Mike Weldon, University of Cambridge, using an ABI Procise 494HT Protein Sequencer. This analysis was limited to 5 residues, which is the minimum number of amino acids required to unequivocally identify a proteolytic cleavage site. N-terminal sequences of spot 7 (pI 4, ~23 kDa) and spot 10 (pI 3, ~20 kDa) are shown in Figure 2A. The amino acid sequence of spot 10 matched to the intact N-terminus of the LC while, differently to what observed in the anti-HC Western blot analysis, no heavy chain sequences were detected. A possible explanation for this result is that spot 10 contains a low abundance of HC fragments (a faint signal was observed in the anti-HC Western blot analysis) or that their N-terminus could be blocked either naturally or as a result of sample processing. In fact, it has been demonstrated that many proteins cannot be directly sequenced by Edman degradation because they have a blocked N-terminal residue.13 In the case of spot 7 we found the N-terminal sequence STKGP corresponding to position 119–123 of the CH1 domain. Most interestingly, cleavage sites at the interface between VH and CH1, very close to the STKGP sequence, were previously identified in mAb 2G12 by 2 other research groups11,14 (Fig. 2B), suggesting that this antibody region might be particularly susceptible to proteolytic cleavage. The molecular weight of the species in spot 7 (~23 kDa) indicates that the C-terminal sequence is likely to be localized in the inter-domain region C\textsubscript{H}2-C\textsubscript{H}3 (a schematic illustration is found in Figure 2A), the same region where cleavage sequences had been previously identified by Hehle and colleagues.11

In conclusion, 2-DE analysis coupled with N-terminal sequencing proved to be a powerful method to characterize the products of antibody degradation in plants and identify putative sequence features responsible for it. In this work, we identified a novel mAb H10 proteolytic cleavage site within the antibody HC region, which is located at the interface between VH and CH1 domains and is largely exposed to the solvent. This result strengthens the hypothesis that different antibodies produced in plants share common cleavage events, and that these events...
preferably take place in solvent accessible, inter-domain regions of antibody molecules.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
1. Paul M, Ma JK-C. Plant-made pharmaceuticals: leading products and production platforms. Biotechnol Appl Biochem 58:58-67; PMID:21446960; http://dx.doi.org/10.1002/bab.6
2. Faye L, Boudlous A, Benchabane M, Gommed V, Michaud D. Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. Vaccine 2005; 23:1770-8; PMID:15734039; http://dx.doi.org/10.1016/j.vaccine.2004.11.003
3. Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res 2008; 36:D320-5; PMID:17991683; http://dx.doi.org/10.1093/nar/gkm954
4. Smalle J, Vierstra RD. The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 2004; 55:555-90; PMID:15377232; http://dx.doi.org/10.1146/annurev.arplant.55.031903.141801
5. Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E, Donini M. Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. Plant Biotechnol J 2009; 7:59-72; PMID:18793269; http://dx.doi.org/10.1111/j.1467-7652.2008.00371.x
6. Hehle VK, Drake PM, Ma JK-C, van Doolweerd CJ. Antibody degradation in tobacco plants: a predominantly apoplastic process. BMC Biotechnol 2011; 11:128; PMID:22208820; http://dx.doi.org/10.1186/1472-6750-11-128
7. Sharp JM, Doran PM. Characterization of monomolecular antibody fragments produced by plant cells. Biotechnol Bioeng 2001; 73:338-46; PMID:11320504; http://dx.doi.org/10.1002/bab.1067
8. De Muynck B, Navarre C, Bourtier M. Production of antibodies in plants: status after twenty years. Plant Biotechnol J 2015; 8:356-75; PMID:24220303; http://dx.doi.org/10.1016/j.pbi.2013.06.009
9. De Muynck B, Navarre C, Konare E. Different subcellular localization and glycosylation for a functional antibody expressed in Nicotiana tabacum plants and suspension cells. Transgenic Res 2009; 18:467-82; PMID:19140023; http://dx.doi.org/10.1007/s11248-008-9240-1
10. Hehle VK, Lombardi R, van Doolweerd CJ, Paul MJ, Di Micco P, Morea V, Benvenuto E, Donini M, Ma JK-C. Site-specific proteolytic degradation of IgG monoclonal antibodies expressed in tobacco plants. Plant Biotechnol J 2015; 13:235-45; PMID:25283551; http://dx.doi.org/10.1111/pbi.12266
11. De Cark M, Tanino B, Capodicasa C, Villani ME, Salzano AM, Scaroni A, Raschella G, Benvenuto E, Donini M. Proteome changes induced by c-myc silencing in human chronic myeloid leukemia cells suggest molecular mechanisms and putative biomarkers of hematopoietic malignancies. J Proteomics 2014; 96:209-22; PMID:24220303; http://dx.doi.org/10.1016/j.jprot.2013.10.040
12. Leone JW, Hampton B, Fowler E, Moyer M, Krishna RG, Chin CCQ. Removal of N-terminal blocking groups from proteins. Curr Protoc Protein Sci 2011; Chapter 11:Unit11.7; PMID:21400688
13. Niemer M, Mehofer U, Torres Acosta JA, Verdianz M, Henkel T, Loos A, Strasser R, Maresch D, Rademacher T, Stenklleller H, et al. 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. Biotechnol J 2014; 9:493-500; PMID:24478053; http://dx.doi.org/10.1002/biot.201300207