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An Investigative Comparison: Plant-Based MAbs for Hep B Surface Antigen and MAbs from Mice

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Regulatory agencies such as the FDA require the structure and amino acid sequence characterization of recombinant monoclonal antibodies (MAbs) to grant marketing approval.^{1,2} Characterizing such complex, inherently heterogeneous molecules is a significant analytical challenge that requires a broad array of physicochemical tests.³

Mass spectrometry (MS) is an essential tool for characterizing protein identity, functions, substrate specificity and amino acid sequence (AAS) of recombinant MAb biotherapeutics⁴ as it complements, or in some cases supersedes the utility of traditional biological methods. For some of the most important proteomic applications, the high sensitivity and accuracy provided by modern MS has allowed the unequivocal protein characterization.⁵ It also has the capacity to provide valuable information for drug discovery

and quality control of molecules for human use.

Recombinant MAbs are used in diagnostics, therapeutics, as well as reagents for vaccine production—being the predominant biotherapeutic protein under development today.⁶ The CB.Hep-1 is an IgG 2b MAb routinely used as a reagent for the Cuban Hepatitis B vaccine production.⁷ However, due to the current biosafety animal-use constraints, it has been expressed and produced in tobacco plants: plant-derived antibody (plantibody) HB-01.^{8,9}

Utilizing plants to synthesize foreign proteins for human and animal uses has been done for more than two decades. However several differences have been found between vegetable and mammal proteins because codon, amino acid usage, and also glycosylation patterns differ between them.¹⁰

For this reason, a lysine-aspartic acid-glutamic acid-leucine peptide (KDEL)¹¹ used as endoplasmic reticulum (ER) retention signal, was inserted in both plantibody amino acid chains to guarantee the proper glycosylation in the plant ER and Golgi complex. The KDEL signal is sufficient to confer variable extents of accumulation in the plant ER and increased stability when added to proteins destined to the vacuole¹²⁻¹⁵ or secretion¹⁶ of cytosolic proteins introduced into the secretory pathway by the addition of a signal peptide.^{17,18}

This is a unique opportunity to survey the expression system influence on the molecule, since the comparison object is the same antibody produced

in mice and plants. No reports have ever been prepared comparing sequences of plantibodies and their counterparts. Because of this, our work is scientifically notable. Despite the benefits of expressing proteins in plants, probable complementarity-determining region (CDR), framework and constant fragment (C) modifications might compromise the antigen recognition and antibody functions.

The main objective of this work was to determine and characterize the AAS of both antibodies and compare them by MS technique. This is one of the first demonstrations to confirm that the expression and production technology of antibodies in plants (tobacco) does not modify the plantibody AAS, the antigen recognition capacity, affinity constant (K_{aff}), and functions.

Materials and Methods

MAb CB.Hep-1

Purification and Quantification

A total amount of 10⁶ cells were inoculated intraperitoneally in each mineral oil primed BALB/c mouse. Cell viability was measured by trypan blue exclusion method.¹⁹ The ascites were harvested, filtered and submitted to two ammonium sulfate precipitations. The material was centrifuged at 4,800 xg for 20 min at 4°C. The dissolved pellet was desalted by gel filtration chromatography in Sephadex G-25 coarse (GE Healthcare, Amersham Biosciences, Uppsala, Sweden) and phosphate-buffered saline 100 mM (PBS) pH 8.0 for the mobile phase.

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Then the desalted material was purified by rProtein A Sepharose FF affinity chromatography (GE Healthcare) using 100 mM PBS, pH 8.0 absorption buffer and 0.1 M citric acid, pH 3.0 as elution buffer. Immediately after, sample buffer was exchanged to 20 mM Tris/150 mM NaCl, pH 7.6 by gel filtration chromatography in Sephadex G-25 coarse. Finally, MAb CB.Hep-1 was quantified according to Valdés *et al.*²⁰

Plantibody HB-01

Purification and Quantification

The molecule purification and quantification was done according to Valdés *et al.*⁹

SDS-PAGE and PNGaseF

Deglycosylation

The SDS-PAGE (12.5%) under reduced conditions was done for antibody chain separations according to Laemmli,²¹ and 25 µg of protein were applied on each lane after a deglycosylation with PNGaseF (New England BioLabs, Ipswich, Mass., USA).

Protein Digestion and

Peptide Extraction Protocol

The tryptic protein digestion followed a recently published protocol.²² Gel digestions with lysyl endopeptidase (LEP) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), endoproteinase Asp-N (Boehringer Ingelheim GmbH, Ingelheim, Germany), and quimotrypsin (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) were done along with a standard method reported for tryptic digestions. Peptides were recovered and desalted with ZipTip C18 columns (Millipore Corp., Billerica, Mass., USA) followed by an elution step in 60% acetonitrile/1% formic acid. Finally, solutions were mixed and loaded into the gold-coated capillaries.

Mass Spectrometry

The low-electrospray ionization (ESI)/MS and MS/MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QToF2 (Waters Corp., Milford, Mass., USA) fitted with a Z-spray nanoflow ESI source. Other measuring conditions and data processing

were done according to González *et al.*²³

Results and Discussion

It is not possible to fully control and predict the outcome of genetic modifications of organic MS. Concerning the receipt organism, the basis of this incapability can be traced to the following aspects: a) the complexity of even the simplest organic MS makes it impossible to predict the full range of effects resulting from changing even one single gene; b) recombinant DNA manipulations induce mutations at random locations within the genome of the recipient organism; and c) although structural genetic information is universal in its meaning, regulatory genetic information differs in significance depending on the cell and organism type into which it is introduced.

In contrast, concerning the introduced gene, it could be observed in different events that might compromise the appropriate expression of it. For example, when a mammalian gene is introduced in a plant genome: a) possible

gene silencing events occur; b) the codon usage could have a huge impact in the sequence and also in the expressed protein tri-dimensional conformation; and c) the genetic recombination, mutations and transposomes could affect the final DNA sequence, the amino acid sequence, and of course the functionality of the expressed protein.

Nowadays the expression and production of IgGs in plants for human and animal uses is a routine scientific activity.⁸ Nevertheless, several disparities between vegetable and mammal proteins have been found due to differences in molecular mechanisms between both types of cells¹⁰ such as in the use of the synonymous codons which have been extensively analyzed in bacteria, yeast, and higher eukaryotes.²⁴ Eighteen of the amino acids are encoded by more than one codon while methionine (M) and tryptophan (W) have only one codon.

The quaternary structure of an IgG molecule is composed of two light and two heavy amino acid chains which are connected by disulphide bridges and other interactions. The molecule is functionally divided in fragment of antigen binding (Fab) and Fc fragments.

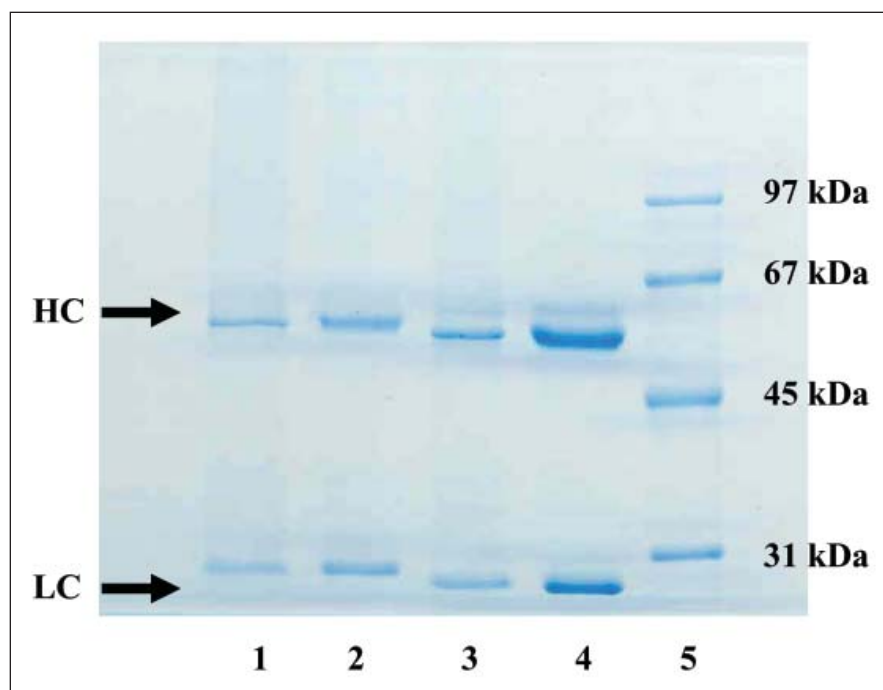


Figure 1. Coomassie stained SDS-PAGE (12.5%, reduced conditions). Lane 1: plantibody HB-01 deglycosylated. Lane 2: plantibody HB-01. Lane 3: MAb CB.Hep-1 deglycosylated. Lane 4: MAb CB.Hep-1. Lane 5: molecular weight pattern (phosphorylase B: 97 kDa; bovine seroalbumin: 67 kDa; ovalbumin: 45 kDa; carbonic anhydrase: 31 kDa). HC: heavy chain; LC: light chain.

DIVMSQSPSSLAVSVGEKVALSCKSSQSLLYLNNHKNYLAWFQQKPG
 QSPKLLIYWASTRDSGVPDRFTGSGSGTDFLMISSVKAEDLAVYYCQ
 QYYNYPYTFGGGKLEIKRADAAPTVISFPPSSEQLTSGGASVVCFLN
 NFYPKIDINVKWKIDGSRQNGVLNSWTDQDQSKDSTYSMSSTLTLTK
 DEYERHNSYTCETHKTSTSPIVKSFNREK

Mouse monoclonal antibody CB.Hep-1 Light Chain

EVKLDETGGGLVQPGRPMKLSBVASGFTFSDFWMNWVRQSPEKGLE
 WVAQIRDKPDNYAIYSESVKGRFTISRDDSRSSVFLQMNSLRPEDT
 GIYYBTAGFDYWGQGTTLTVSSAKTTPPSVYPLAPBGDTTGSSVTL
 GBLVKGYFPESVTVTWNSGSLSSSVHTFPALLQSGLYTMSSSVTPSS
 TWPSQTVTBSVAHPASSTTVDKKLEPSGPISTINBPBPBKEBHKBPAP
 NLEGGPSVFIFFPNKIDVLMISLTPKVTBVVDVSEDDPDVQISWVFN
 NVEVHTAQQTTHREDYNSTIRVVSTLPIQHQQDWMMSGKEFKBKVNNK
 DLPSPPIERTISKIKGLVRAPQVYILPPPAEQLSRKDVSLTLBVGFNPGD
 ISVEWTSNGHTEENYKDTAPVLDSDGSYFIYKLNMKTSKWEKTDSE
 SBNVRHEGLKNYYLKKTISRSPGK

Mouse monoclonal antibody CB.Hep-1 Heavy Chain

MDIVMSQSPSSLAVSVGEKVALSCKSSQSLLYLNNHKNYLAWFQQKPGQ
 SPKLLIYWASTRDSGVPDRFTGSGSGTDFLMISSVKAEDLAVYYCQQYY
 NYPYTFGGGKLEIKRADAAPTVISFPPSSEQLTSGGASVVCFLNNFYPK
 INVKWKIDGSRQNGVLNSWTDQDQSKDSTYSMSSTLTLTKDEYERHNS
 YTCEATHKTSTSPIVKSFNREKSRKDEL

Plantibody HB-01 Light Chain

_EVKLDETGGGLVQPGRPMKLSBVASGFTFSDFWMNWVRQSPEKGLE
 WVAQIRDKPDNYAIYSESVKGRFTISRDDSRSSVFLQMNSLRPEDTGIY
 YBTAGFDYWGQGTTLTVSSAKTTPPSVYPLAPBGDTTGSSVTLGBLVK
 GYFPESVTVTWNSGSLSSSVHTFPALLQSGLYTMSSSVTPSSWPSQT
 VTBVAHPASSTTVDKKLEPSGPISTINBPBPBKEBHKBPAPNLEGGP
 SVFIFFPNKIDVLMISLTPKVTBVVDVSEDDPDVQISWVFNVEVHTAQ
 TQTHREDYNSTIRVVSTLPIQHQQDWMMSGKEFKBKVNNKDLPSPIERTI
 SKIKGLVRAPQVYILPPPAEQLSRKDVSLTLBVGFNPGDISVEWTSNGH
 TEENYKDTAPVLDSDGSYFIYKLNMKTSKWEKTDSEFBNVRHEGLKNY
 YLKKTISRSPGKSRKDEL

Plantibody HB-01 Heavy Chain

Figure 2. Plantibody HB-01 and MAb CB.Hep-1 amino acid sequences. CDR, frameworks and Fc.

Table 1. Peptides obtained by trypsin digestion of the MAb CB.Hep-1 light chain (m: mass; z: electric charge).

Fractions	m/z Theoretical	m/z Experimental	Sequence and Localization
1	(1+) 676.33	676.32	¹⁵⁶ IDGSER ¹⁶¹
	(1+) 711.29	711.32	¹⁹⁰ DEYER ¹⁹⁴
2	(1+) 333.19	333.21	¹⁵⁴ WK ¹⁵⁵
	(1+) 523.26	523.28	²¹⁴ SFNR ²¹⁷
	(1+) 745.35	745.36	⁶¹ DSGVPDR ⁶⁷
3	(2+) 674.29	674.30	¹⁹⁵ HNSYTC*EATHK ²⁰⁵
	(1+) 1347.58	1347.57	¹⁹⁵ HNSYTC*EATHK ²⁰⁵
	(1+) 926.37	926.38	²¹⁴ SFNRNEC* ²²⁰
4	(1+) 588.34	588.35	¹⁴⁹ DINVK ¹⁵³
	(1+) 677.37	677.38	¹⁹ VALSC*K ²⁴
5	(1+) 832.48	832.49	²⁰⁶ TSTSPIVK ²¹³
6	(1+) 502.32	502.33	¹¹⁰ LEIK ¹¹³
7	(1+) 990.49	990.48	¹⁵⁴ WKIDGSR ¹⁶¹
8	(2+) 702.37	702.38	²⁵ SSQSLLYLNNHK ³⁶
	(1+) 1403.73	1403.72	²⁵ SSQSLLYLNNHK ³⁶
9	(2+) 702.37	702.36	²⁵ SSQSLLYLNNHK ³⁶
	(2+) 796.37	796.39	¹⁶² QNGVLNSWTDQDSK ¹⁷⁵
10	(2+) 796.87	796.86	¹⁶² QDGVLSWTDQDSK ¹⁷⁵
11	(2+) 767.87	767.87	¹⁷⁶ DSTYSMSSTLTLTK ¹⁸⁹
	(1+) 1534.73	1534.74	¹⁷⁶ DSTYSMSSTLTLTK ¹⁸⁹
12	(2+) 796.87	796.86	¹⁶² QDGVLSWTDQDSK ¹⁷⁵
	(2+) 917.47	917.49	¹ DIVMSQSPSSLAVSVGEK ¹⁸
13	(2+) 1114.00	1114.02	¹⁷⁶ DSTYSMSSTLTLTKDEYER ¹⁹⁴
	(2+) 896.46	896.48	³⁷ NYLAWFQQKPGQSPK ⁵¹
14	(3+) 957.76	957.75	⁸⁶ AEDLAVYYC*QQYYNYPYTFGGGK ¹⁰⁹
	(2+) 1436.13	1436.16	⁸⁶ AEDLAVYYC*QQYYNYPYTFGGGK ¹⁰⁹
15	(2+) 917.95	917.93	⁶⁸ FTGSGSGTDFLMISSVK ⁸⁵
	(1+) 122.63	122.66	⁵² LLIYWASTR ⁶⁰
16	(3+) 1243.29	1243.27	¹¹⁴ RADAAPTVISFPPSSEQLTSGGASVVC*FLNNFYPK ¹⁴⁸
17	(3+) 1191.25	1191.27	¹¹⁵ ADAAPTVISFPPSSEQLTSGGASVVC*FLNNFYPK ¹⁴⁸

Fab comprises the variable region where the antigen recognition takes place thanks to CDRs and frameworks. By contrast, Fc comprises the constant region usually called the effector region.²⁰ Therefore, a deep molecular characterization is needed in order to determine the AAS, to find out if there are differences between both antibodies, and determine if they have any impact on the antibody specificity and Kaff.

The ESI/MS has been used to elucidate the structure of recombinant DNA-derived biotherapeutics.²⁵⁻²⁸ However, with the high molecular weight, complex structure and large number of potential mass isoforms, MS presents significant challenges to the MS analysis of recombinant MAb.³ Both the light and heavy chains were isolated by SDS-PAGE for protein sequencing by MS. The purified samples were submitted for a previous deglycosylation protocol with PNGaseF (Figure 1). This was due to the sugar interferences in the MS results observed in previous experiments

where antibody chain separations were performed by high liquid performance chromatography (HPLC) (data not shown).

The sample purities were, in all cases, more than 95%, according to SDS-PAGE (Figure 1). Plantibody HB-01 and MAb CB.Hep-1 light chains were completely verified and compared. Meanwhile, only 94% of the heavy chains were sequenced due to peptide recovery difficulty from SDS-PAGE gel. The methionine residue that initiates the translation process in mammals was eliminated from MAb CB.Hep-1 AAS. Thus, the light chain begins with an aspartic acid residue. Unexpectedly, this residue is conserved in plantibody HB-01 sequence, probably as a consequence of a post-translational modification absence.

The presence of methionine initiating the plantibody HB-01 heavy chain was not observed since it was implicated in the nonverified sequence (Figure 2). Besides, a 100% of coincidence was observed in the rest of variable region

sequences among four antibody chains.

Tables 1–4 relate the recovered peptides MS data after in-gel digestions. For the plantibody HB-01 light chain, the Asp-N and quimotrypsin digestion generated two and one peptides respectively: ¹⁹¹DEYERHNSYTC*EA THKTSTSPIVKSFNREK*KDEL²²⁵, ¹⁹¹DEYERHNSYTC*EATHKTSTSPIV KSFNREK*KDE²²⁴ for Asp-N, and ²¹⁶NRNEK*(K/Q)D(K/Q)DEL²²⁵ for quimotrypsin.

The tetrapeptide KDEL is commonly found at the ER C-terminus of soluble proteins. It contributes to their localization by interacting with a receptor that recycles between the Golgi complex and the ER.

This signal increases stability when it is added to proteins destined to secretion.¹⁵ Thus, it was necessary to insert a KDEL DNA coding sequence into plantibody HB-01 DNA sequence to guarantee the pathway through ER and Golgi compartments to achieve a proper glycosylation⁸ which was verified

Table 2. Peptides generated by trypsin digestion of the plantibody HB-01 light chain.

Fraction	m/z Theoretical	m/z Experimental	Sequence and Localization
2	(1+) 677.37	677.37	²⁰ VALSC*K ²⁵
2	(1+) 1347.58	1347.58	¹⁹⁶ HNSYTC*EATHK ²⁰⁶
3	(1+) 832.48	832.48	²⁰⁷ TSTSPIVK ²¹⁴
3	(1+) 502.32	502.34	¹¹¹ LEIK ¹¹⁴
4	(1+) 1403.73	1403.73	²⁶ SSQSLLYLNNHK ³⁷
5	(1+) 1591.74	1591.74	¹⁶³ QNGVLNSWTDQDSK ¹⁷⁶
6	(1+) 1534.73	1534.73	¹⁷⁷ DSTYSMSSTLTLTK ¹⁹⁰
6	(1+) 1980.95	1980.98	¹ M(ox)DIVMSQSPSSLAVSVGEK ¹⁹
7	(1+) 1964.97	1964.96	¹ MDIVMSQSPSSLAVSVGEK ¹⁹
7	(1+) 1791.92	1791.92	³⁸ NYLAWFQQKPGQSPK ⁵²
8	(1+) 1834.89	1834.92	⁶⁹ FTGSGSGTDFTLMISSVK ⁸⁶
8	(1+) 2871.26	2871.25	⁸⁷ AEDLAVYYC*QQYYNYPYTFGGGK ¹¹⁰
8	(1+) 1122.63	1122.60	⁵³ LLIYWASTR ⁶¹

Table 3. Peptides generated by LEP digestion of the plantibody HB-01 light chain.

Fraction	m/z Theoretical	m/z Experimental	Sequence and Localization
2	(1+) 677.37	677.38	²⁰ VALSC*K ²⁵
3	(1+) 832.48	832.50	²⁰⁷ TSTSPIVK ²¹⁴
3	(1+) 502.32	502.35	¹¹¹ LEIK ¹¹⁴
4	(1+) 2039.85	2039.92	¹⁹¹ DEYERHNSYTBATHK ²⁰⁶
5	(1+) 1403.73	1403.80	²⁶ SSQSLLYLNNHK ³⁷
6	(1+) 2249.04	2249.14	¹⁵⁷ IDGSERQNGVLNSWTDQDSK ¹⁷⁶
7	(1+) 1534.73	1534.80	¹⁷⁷ DSTYSMSSTLTLTK ¹⁹⁰
9	(1+) 2871.26	2871.37	⁸⁷ AEDLAVYYC*QQYYNYPYTFGGGK ¹¹⁰

Table 4. Peptides generated from the digestion with trypsin, Asp-N, and LEP of the MAb CB.Hep-1 and plantibody HB-01 heavy chain.

Fraction	m/z Theoretical	CB.Hep-1 m/z Experimental	HB-01 m/z Experimental	Sequence and Localization
1	(2+) 827.93	827.93	827.95	⁴ LDETGGGLVQGRPMK ¹⁹
2	(2+) 1162.54	1162.50	1162.56	²⁰ LSC*VASGF ²¹ TSDFWMNWVR ³⁸
3	(1+) 571.30	571.33	571.30	³⁹ QSPEK ⁴³
4	(2+) 536.30	536.35	536.33	⁴⁴ GLEWVAQIR ⁵²
5	(2+) 896.43	896.43	896.43	⁵³ DKPDNYAIYYSESVK ⁶⁷
6	(3+) 1135.07	1135.05	1135.06	⁵⁶ DNYAIYYSESVKGRFTISR ⁷⁴
7	(3+) 660.97	660.98	661.00	⁷⁵ DDSRSSVFLQMNSLRPE ⁹¹
8	(2+) 641.28	641.28	641.31	⁹² DTGIYYC*TAGF ¹⁰²
9	(3+) 1009.16	1009.14	1009.18	¹⁰³ DYWGQGTTLTVSSAKTTTPSVYPLAPGC*G ¹³¹
10	(2+) 1432.21	1432.18	1432.21	¹¹⁸ TTPSVYPLAPGC*GDTTGSSVTLGC*LVK ¹⁴⁵
11	(4+) 1714.40	1714.40	1714.38	¹⁴⁶ GYPESVTVTWNSGLSSVHTFPALLQSGLYTMSSSVTVPSS ¹⁶⁰ WPSQTVTC*VAHPASSTTVDK ²¹⁰
12	(2+) 947.97	948.00	947.99	²¹² LEPSGPISTINPC*PPC*K ²²⁸
13	(2+) 1084.56	1084.64	1084.56	²³³ C*PAPNLEGGPSVFIFPPNIK ²⁵²
14	(2+) 558.82	558.86	558.83	²⁵³ DVLMISLTPK ²⁶²
15	(4+) 1010.24	1010.33	1010.27	²⁶³ VTC*VVVDVSEDDPDVQISWFVNNVEVHTAQTQTHR ²⁹⁷
	(2+) 499.23	499.72	499.70	²⁹⁸ EDYDSTIR ³⁰⁵
16	(3+) 609.31	609.36	609.32	³⁰⁶ VVSTLPIQHQDWMSGK ³²¹
17	(2+) 463.75	463.79	463.76	³³¹ DLPSPIER ³³⁸
18	(3+) 778.12	778.19	778.09	³⁴⁵ GLVRAPQVYILPPPAAEQLSRK ³⁶⁵
19	(3+) 1162.21	1162.32	1162.30	³⁶⁶ DVSLTC*LVVGFNPGDISVWETSNGHTEENYK ³⁹⁶
19	(2+) 939.44	939.44	939.45	³⁹⁷ DTAPVLDSGDGSIYFIYSK ⁴¹³
20	(1+) 505.28	505.28	505.27	⁴¹⁴ LNMK ⁴¹⁷
21	(1+) 335.19	335.21	335.20	⁴¹⁸ TSK ⁴²⁰
22	(1+) 462.24	462.28	462.26	⁴²¹ WEK ⁴²³
23	(1+) 550.24	550.30	550.26	⁴²⁴ TDSFSC*NVR ⁴³²
	(2+) 832.40	832.38	832.36	⁴²⁴ TDSFSC*NVRHEGLK ⁴³⁷
	(1+) 700.37	700.37	700.38	⁴³⁸ NYYLK ⁴⁴²
24	(1+) 476.28	476.32	476.32	⁴⁴⁴ TISR ⁴⁴⁷
	(2+) 423.24	423.26	423.25	⁴⁴⁴ TISRSPGK ⁴⁵¹
25	(1+) 376.16	-----	376.20	⁴⁵⁴ KDEL ⁴⁵⁷

by ESI/MS spectra (Figures 3A and 3B). Hence, C-terminus plantibody HB-01 sequence for both chains contains the fragment SRKDEL. They represent approximately 1 kDa more with respect to CB.Hep-1 molecular mass and may be the responsible factor of migration differences observed in SDS-PAGE, even in deglycosylated protein (Figure 1).

Surprisingly, the influence of the host was almost absent, taking into account that only slight expected amino acid differences were found in the molecule.

These differences do not modify the antigen recognition capacity (specificity), Kaff and immunopurification behavior according to Valdés *et al.*²⁹ Therefore, the expression and plant production technology is a validated alternative for producing therapeutic MAb nearly equal to its mouse counterparts in AAS. Nevertheless, this conclusion is not absolute for all the plant-expressed molecules because differences in codon and amino acid usage are not the same between plant and mammal systems.

Conclusions

The AAS differences determined by SDS-PAGE-ESI/MS between both molecules were concentrated at the C and N-terminus regions. The C-terminus modification matches the expected sequence introduced for the ER allocation and does not modify the specificity, Kaff, and plantibody HB-01 functions.

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Figure 3A. The ESI MS/MS spectra of the $[mass(m)+2H]^{2+}$ signal at 504.21 corresponding to the C-terminus peptide of the plantibody HB-01 light chain.

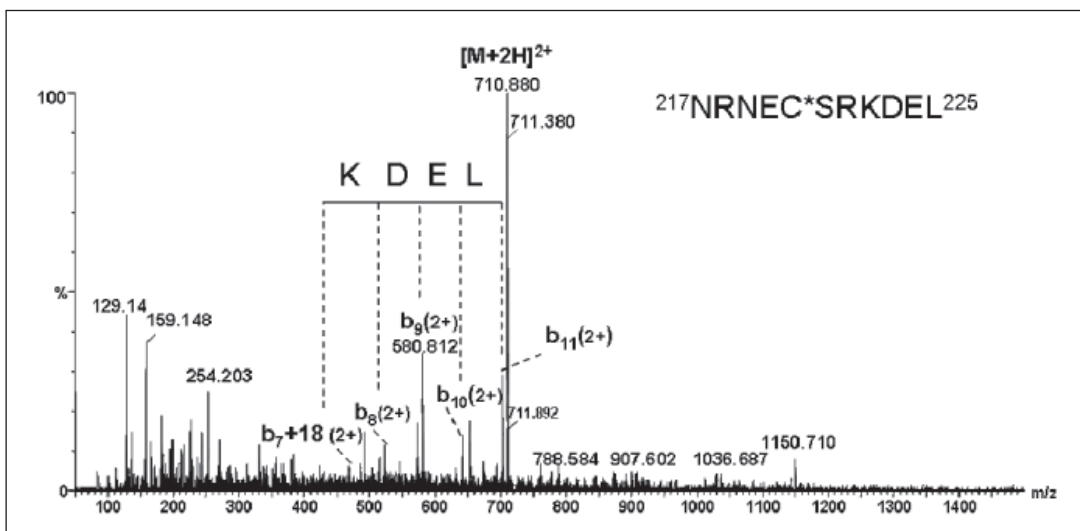
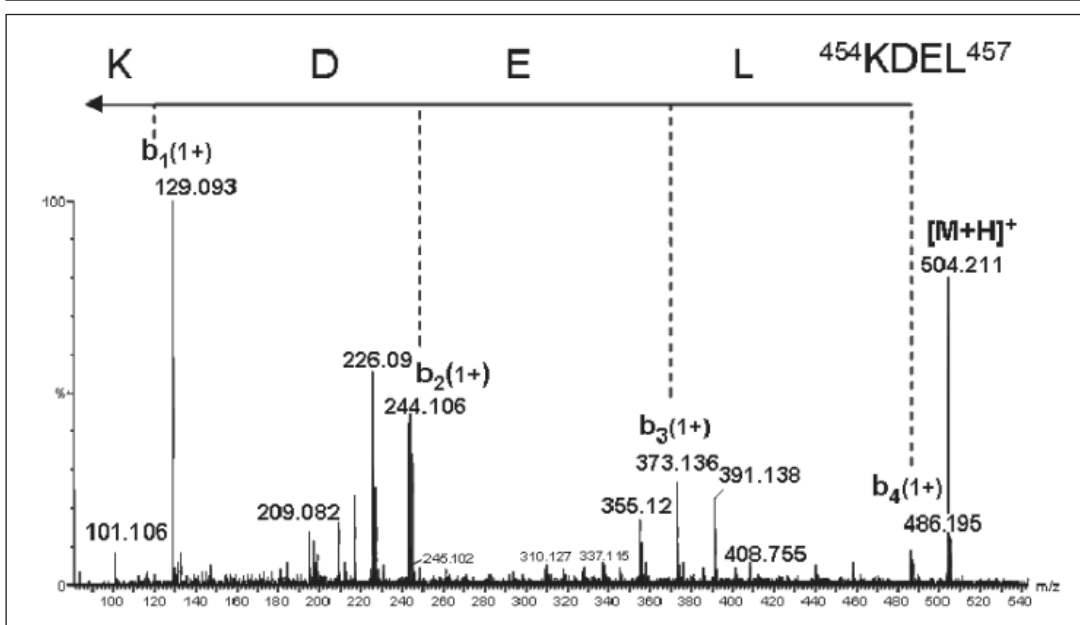


Figure 3B. The ESI MS/MS spectra of the $[M+H]^+$ signal at 710.88 corresponding to the C-terminus peptide of the plantibody HB-01 heavy chain (H: proton; C*: carbamidomethylated cysteine).



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