A publication of
The Williamsburg BioProcessing Foundation

Spring 2007
ISSN 1538-8786

BioProcessing JOURNAL

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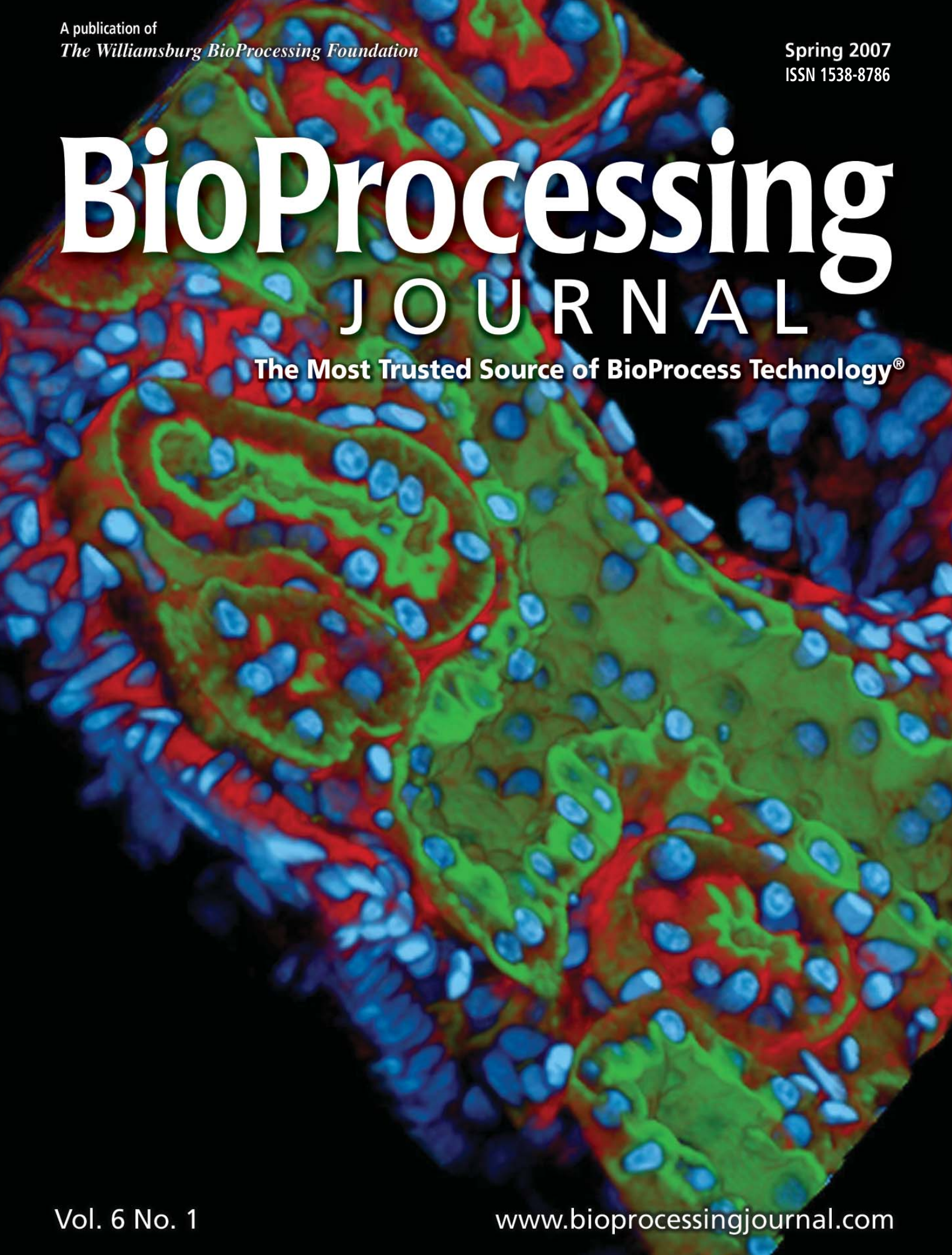

Vol. 6 No. 1

www.bioprocessingjournal.com

Effects of Tobacco Extract and Temperature On the Stability of the Monoclonal Antibody CB.Hep-1 Expressed in Transgenic Tobacco Plants

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Proteins are widely used in research, medicine and industry, but its extraction from their natural sources can be difficult, tedious and expensive. Therefore, a simple and inexpensive system that allows large-scale production of safe recombinant proteins will always be highly desirable. Traditional production systems that use microbial, insect and mammalian cell cultures have drawbacks, in terms of cost, scalability and product safety.[1-5]

Several studies have shown that molecular farming in plants has many practical, economic, and safety advantages as compared to these conventional methods. Thus, the use of plants for recombinant protein synthesis is gaining wide acceptance.

The first pharmaceutically-relevant protein made in plants was the human growth hormone which was expressed in transgenic tobacco plants in 1986.[6] Since then, many other animal and human proteins have been produced in a diverse range of crops. In 1989, Hiatt *et al.* expressed the first antibody in tobacco plants, showing that plants could assemble complex glycoproteins.[7] The structural authenticity of plant-derived recombinant proteins was confirmed in 1992 when plants were used to produce an experimental vaccine, the Hepatitis B virus surface antigen.[8]

The protein-synthesis pathway is highly conserved between plants and animals. Hence, human transgenes that are expressed in plants yield proteins with almost identical amino acid sequences to their native counterparts. Nevertheless, there are important differences in the post-translational modifications. The main difference concerns the synthesis of glycan side chains. Plant-derived recombinant proteins tend to lack the terminal galactose and sialic acid residues that are normally found in mammals but have the carbohydrate group (α 1,3) fucose, which has a (1,6) linkage in animal cells, and (β 1,2) xylose that is absent in mammals. These glycan structure differences could change the activity, biodistribution and stability of recombinant proteins, as compared to the native forms.[9]

Tobacco plants have an established history in molecular farming.[10] The main advantages of this plant include the mature technology for gene transfer expression, and the high biomass yield (more than 100,000 kg per hectare for close-cropped tobacco). However, leaf crude extract contains a high amount of proteases that act during storage, breaking the rest of the proteins down into peptides in a temperature-dependant way;[11,12] a major reason why the composition of leaf crude extract could represent a complex mixture to purify unstable and low-level expressed proteins.[13]

Generally, these proteins are produced at low levels, typically less than 0.1% of total soluble protein. This level of production probably reflects a combination of poor protein folding and stability. It is also important to minimize browning reactions which can inhibit the activity of recombinant proteins in this environment. The enzymatic browning phenomenon occurs when proteins or peptides react with oxidized phenolic compounds.

Taking into account these ideas, some plant-derived protein stability studies have been carried out in several crops: a) β-glucuronidase (GUS) stability in canola and corn at 10°C (room temper-

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ature); b) elevated temperatures and different moisturizing conditions;[14-16] c) long-term stability of heterologous proteins under nonspecified conditions;[17-19] d) monoclonal antibody (mAb) IgG two-years stability in corn;[20] e) single chain variable fragment (scFv) antibody stability in cereal and peas;[19] f) mAb C5-1 stability in alfalfa;[21] g) mAb stability in potato tubers;[22] and h) one-week stability of a scFv antibody in dried tobacco leaves.[23] Only one problem: they don't work—none of those performed in tobacco were focused in the evaluation of mAb stability in tobacco crude extract at different temperatures.

Based precisely on that fact, we defined a plan to investigate whether proteolytic degradation of the tobacco plant-derived antibody (plantibody) HB-01, used for the immunopurification of the recombinant hepatitis B surface antigen (rHBsAg), occurs in the transgenic tobacco extract.

Materials and Methods

mAb and Plantibody Sources

The mouse B-lymphocyte hybridoma 48/1/5/4 was previously generated by Fontirrochi *et al.*[24] Briefly, it was obtained by fusion of Sp2/0-Ag14 myeloma cells and spleen cells of a BALB/c mouse immunized with HBsAg. This hybridoma secretes an IgG-2b kappa mAb (CB.Hep-1) directed against the "a" determinant of the HBsAg.[25] Transgenic tobacco plants expressing the anti-HBsAg antibody (CB.Hep-1) generated by Ramirez *et al.* [26] were used for this study.

CB.Hep-1 mAb Production By Ascites Method

Cells were cultivated in spinner flasks using RPMI-1640 (Invitrogen Corp., GIBCO, Gaithersburg, Maryland, USA) supplemented with 8% (v/v) of foetal calf serum (FCS) (Invitrogen Corp., GIBCO), 2 mM L-glutamine, 1 mM sodium piruvate, 17 mM sodium bicarbonate, and 25 mg/L of gentamicin. Cells were always maintained at 37°C in 5% CO_2 atmosphere, and the medium was changed every 48 hours. Subsequently, 10^6 cells were inoculated intraperitoneally in each mineral oil-primed BALB/c mouse, and the ascites was harvested, filtered, and submitted to two ammonium sulfate precipitations.

In both 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 (GE Healthcare, Amersham-Biosciences, Uppsala, Sweden) using a BPG 200/750 column (GE Healthcare, Amersham-Biosciences) with a flow rate of 130 cm h^{-1} and phosphate buffered saline (PBS) 150 mM pH 8.0 as mobile phase. The desalted material was then purified by Protein A Sepharose CL-4B fast flow (GE Healthcare, Amersham-Biosciences) using PBS 150, mM pH 8.0 as adsorption buffer and 100 mM citric acid, pH 3.0 as elution buffer. Immediately after this step, the pH of the eluted fractions was neutralized to allow the buffer exchange to 20 mM tris/150 mM NaCl, pH 7.6 by a gel filtration chromatography in Sephadex G-25.[27]

CB.Hep-1 mAb Production by Hollow Fiber Bioreactor (HFB) Method

About 3.8 x 10^9 cells were inoculated into the bioreactors by means of 50 ml syringes at a speed of 10 ml per minute. HFB CP2500 (Unisyn Technologies, Inc., Hopkinton, Mass., USA) was used with a bioreactor of 70 ft^2, 30 kDa of molecular weight cut-off, and an oxygenator (OXY25A). All critical bioprocessing functions were controlled by the UniNet Notifier software system (Honeywell International, Inc., Northford, Conn., USA).

The culture medium used in the extracapillary space was Iscove's Modified Dulbecco Medium (Invitrogen Corp., GIBCO), supplemented with 10% (v/v) of FCS (Invitrogen Corp., GIBCO), 2 mM L-glutamine, 1 mM sodium piruvate, 17 mM sodium bicarbonate, and 25 mg/L of gentamicin. A similar medium formulation was used for the intracapillary space of the bioreactors, but without FCS. The supernatant was purified by expanded bed adsorption chromatography using PBS 150 mM, pH 8.0 as adsorption buffer and 100 mM citric acid, pH 3.0 as elution buffer. The sample buffer was exchanged to 20 mM tris/150 mM NaCl, pH 7.6 by gel filtration chromatography in Sephadex G-25.

Plantibody HB-01 Production

Tobacco plants were grown in natural granulated zeolite. Seedlings were transplanted and leaves were then harvested (at the age of six weeks after transplantation) and then ground. Extraction buffer in a ratio of 400 ml per every kilogram of leaves was used to extract proteins. This material was then processed using a Fitzmill Comminutor (The Fitzpatrick Co., Elmhurst, Illinois, USA) and separated by centrifugation at 1,051.38 xg in a Rina basket centrifuge (Riera Nadeu, N.A., Barcelona, Spain).

Supernatant was centrifuged again at 19,635 xg in a CEPA tubular centrifuge (Carl Padberg GmbH, Lahr, Germany) and simultaneously pumped to the STREAMLINE rProtein A Sepharose column (GE Healthcare, Amersham-Biosciences). The following buffers and flow rates were employed: a) equilibration and wash buffer, PBS 150 mM, pH 8.0 (300 cm h^{-1}); and b) elution buffer, 100 mM citric acid, pH 3.0 (100 cm h^{-1}). Soon after the elution, the pH of the eluted fractions was adjusted to 7.6 and the buffer exchange was carried out by means of a gel filtration chromatography using Sephadex G-25.[27]

Estimation of Specific Antibody Activity by Enzyme-Linked Immunosorbent Assay (ELISA) and Protein Determination

A polystyrene microplate (Corning, Inc., Life Sciences, Lowell, Mass., USA) was coated with 10 µg/well of recombinant HBsAg in 100 mM $NaHCO_3$ buffer for 20 min at 50°C. After this, step samples were added to the plate in 0.05% tween 20 in PBS 150 mM and incubated for 1 hour at 37°C. After several washings with 0.05% (v/v) tween 20/PBS 150 mM, the plate was incubated for 1 hour at 37°C with a horseradish peroxidase conjugate (Sigma-Aldrich, Inc., St. Louis, Missouri, USA). The reaction was then revealed using 100 µl/well of 0.05% (w/v) O-phenylenediamine and 0.015% (v/v) H_2O_2 in citrate buffer, pH 5.0, and stopped with 50 µl/well of 1.25 M H_2SO_4. The absorbance was measured in a Multiskan ELISA reader (Labsystems,

ICN Biomedicals, Helsinki, Finland) using a 492 nm filter.[25] In all cases, the protein concentration was determined using the method described by Lowry *et al.* using bovine serum albumin (BSA) as standard material.[28]

Purity Measured by Sodium Duodecylsulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blotting

Sample purity was analyzed by gel electrophoresis on a 12.5% (w/v) SDS-PAGE[29] followed by Coomassie staining. The purity of the bands was determined by means of the Molecular Analyst software, version 1.4.1 (Bio-Rad Laboratories, Hercules, California, USA). Samples analyzed by gel electrophoresis on a 12.5% SDS-PAGE were transferred to nitrocellulose and immunoblotted using a peroxidase-conjugated goat anti-mouse IgG and diaminobenzidine as color substrate for identification of immunoglobulins.[30]

Endoglycosidase Digestion of Glycoproteins

Enzymatic deglycosylation was carried out either with peptide-N-glycosidase (PNGase) F or PNGase A.

For deglycosylation with PNGase F, proteins were denatured at 70˚C for 10 min in 1% (v/v) SDS and 5% (v/v) 2-mercaptoethanol. Each sample was incubated at 37˚C for 2 hours with 5 U mg^{-1} of PNGase F (Roche Applied Science, Penzberg, Germany). After enzymatic digestion, deglycosylated protein was precipitated by three volumes of cold ethanol addition, recovering the N-glycan pool from the supernatant.

For deglycosylation with PNGase A, protein was first digested with 10 µg pepsin in 1 ml of 0.01 N HCl (pH 2.0) for 48 hours at 37°C. The reaction mixture was neutralized with 3.2% NH_4OH, heated at 100°C for 5 minutes, and then freeze-dried. The sample was deglycosylated with PNGase A (Roche Applied Science, Penzberg, Germany), at pH 5.0 at 37°C for 24 hours. The substrate/enzyme ratio was 200 µg of IgG per 50 µU of PNGase A. The N-glycans were purified with a GlycoClean H cartridge (ProZyme, San Leandro, California, USA), which was equilibrated with 3 ml of solvent B (50% [v/v] acetonitrile [aq] containing 0.1% Trifluoroacetic acid [TFA]) and 6 ml of solvent A (5% [v/v] acetonitrile [aq] containing 0.1% TFA). The column was washed with 3 ml of distilled water and 3 ml of solvent A. The N-glycans were eluted with 4 x 0.5 ml of solvent B.

Fluorophore Labeling of Oligosaccharides

Labeling reactions were carried out by the fluorophore tag amimobenzoic acid (2AB).[31] Briefly, glycans were dissolved in 5 µl of 2AB (0.35 M) in dimethyl sulfoxide (DMSO)/glacial acetic acid (7/3, v/v) containing sodium cyanoborohydride (1 M) and incubated at 60°C for 2 hours. Subsequently, labeling mixture was applied to a strip of 3MM chromatographic paper (10 cm length x 3 cm width). Ascending paper chromatography was performed using acetonitrile at ambient temperature for 15 min in a glass vessel. Finally, the paper was dried and the labeled glycans were eluted using water.

Normal-Phase High Liquid Performance Chromatography (HPLC) of the N-Glycans 2AB-Derivatives

Normal-phase HPLC was carried out using the TSK-GEL Amide-80 column (4.6 x 250 mm) (Tosoh Bioscience, Montgomeryville, Penn., USA).

Gradient conditions of solvent A: 50 mM ammonium formate, pH 4.4 (prepared by adjusting formic acid solution to the required pH using ammonia solution and diluting to give the appropriate concentration in the final solution). Solvent B gradient conditions: acetonitrile 20% (v/v) A at a flow rate of 0.4 ml/min, followed by a linear gradients of 20–53% (v/v) A in 132 min and 53–100% (v/v) A in the next 3 min. Immediately, flow rate was increased to 1 ml/min in 2 min and the column washed with 100% (v/v) A for 5 min before being equilibrated in the initial conditions. Fluorescence was measured at λex: 356 nm and λem: 450 nm.

Matrix-Assisted Laser Desorption Ionization (MALDI)–Mass Spectrometry (MS) of N-Glycan Derivatives

Mass spectra were recorded with the autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, Mass., USA) operated in the positive ion reflectron mode at 20 kV accelerating voltage with pulsed ion extraction enabled. The lyophilized samples (~5–10 pmol) were re-dissolved in 0.1% (v/v) TFA and analyzed with a matrix, 2,5-dihydroxybenzoic acid (10 mg/ml in 0.1% [v/v] TFA).

Preparation of Non-Transgenic Tobacco Extracts and Sample Incubation

Non-transgenic tobacco plants were grown in a greenhouse under controlled conditions. Seedlings were transplanted at the rate of 15 plants/meter^2. Six weeks after transplanting, leaves were then harvested to be macerated, dropping PBS 150 mM, pH 8.0 with ascorbic acid at 4°C. This material was then separated by centrifugation at 500 xg in a Universal 16 centrifuge (Hettich AG, Bäch, Germany). Ten ml of this extract were mixed with the corresponding antibody: a) CB.Hep-1 produced by ascitic fluid; b) CB.Hep-1 produced by HFB; and c) plantibody HB-01; respectively.

These samples were incubated at 15°C, 25°C, and 37°C in a drying oven (WTB Binder/Brinkmann, Tuttlingen, Germany) and stored at -20°C after each time of incubation (0, 5, 10, 15 and 24 hours) to the moment of the SDS-PAGE and ELISA tests. The mAb CB.Hep-1 produced by ascitic fluid and HFB were mixed to a final concentration of 1 mg/ml, while the plantibody HB-01 was mixed at 200 g/ml of the extract.

Statistical Analysis

Stability of the samples was evaluated by a regression analysis for each temperature and source using the Prism 4.0 software (Graphpad Software, Inc., San Diego, California, USA). Samples were considered stable into tobacco extract at different temperatures when the slope was not significant ($p > 0.05$). Differences in the recovery and purity were compared by the test U of Mann-Whitney (value of medians) using the StatGraphic Plus 5.0 software. The level

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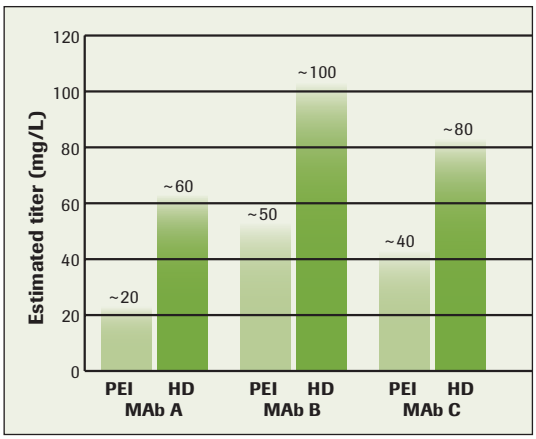

Figure 1: Achieve yields of up to 100 mg/L with FuGENE® HD Transfection Reagent. Results from 1 L shake flask cultures of HEK-293 EBNA cells transfected using FuGENE® HD Transfection Reagent (HD) or a transfection reagent (PEI) from another supplier for production of three different recombinant monoclonal antibodies (A, B, and C). Expression levels were significantly increased using FuGENE® HD Transfection Reagent for all three MAbs, with MAb B achieving approximately 100 mg/L.

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of confidence in this case was also 95% of $p > 0.05$.

Results and Discussion

Recombinant proteins accumulate to high levels in plant cells, and plantibodies are virtually indistinguishable from those produced by hybridoma cells. Several procedures for isolation and purification of full-length antibodies from tobacco have been reported.[32-33] These involve the homogenization of transgenic material which causes the release of proteases and oxidizing agents (*e.g.*, phenols or tannins) from subcellular compartments.[11,34-36] Furthermore, all cell debris must be removed from the crude extract before further processing.

There is some evidence of the stability of recombinant proteins expressed in plants (*e.g.*, GUS stability in canola and corn at 10°C for six months). Nevertheless, when it was left at room temperature, GUS lost up to 20% of its original activity within four days. In addition, for elevated temperatures such as those encountered during dry grinding, this recombinant protein was stable only for two minutes. Finally, reduced GUS stability was found as the moisture content was increased.[14-16]

Long-term stability (about six months) of heterologous proteins in different seed/grain crops has also been demonstrated under unspecified conditions.[17-19] In mAb IgG, two years of stability was reported in corn,[20] scFv antibody stability in cereal and peas,[19] and mAb stability in potato tubers.[21] In contrast, the stability in green leaf material such as tobacco leaves is the more challenging reason for why only a few cases have been reported: mAb C5-1 stability in alfalfa,[22] and one-week stability for a scFv antibody in dried tobacco leaves.[23]

However, there is not much evidence about the stability of recombinant proteins in leaf extracts. The analysis of the influence of the plant extract and temperature in the molecular integrity of recombinant proteins produced by transgenic plants is also a remarkable aspect. This impacts directly into the process recovery, the processing time, and also into the quality of the purified molecule. Considering the potential impact of this aspect on the breakdown of mAb, and the lack of evidence, we have investigated whether proteolytic degradation of the tobacco plantibody HB-01 occurs in the transgenic tobacco extract at different temperatures for 24 hours.

The basic antibody (IgG) structure is a four-polypeptide chain molecule containing two identical light chains and two identical heavy chains. This structure has two antigen binding sites, and both chains contribute to antigen binding, and a fragment-denominated Fc because it is easily crystallized. Glycosylation is an important post-translational modification in this molecule which generates structural micro-heterogeneity between antibody molecules. This should have an important influence on the molecule stability. Antibodies have at least one N-linked carbohydrate added to the constant regions of the heavy chains, but several antibodies also have V-region-associated carbohydrate. The role of the

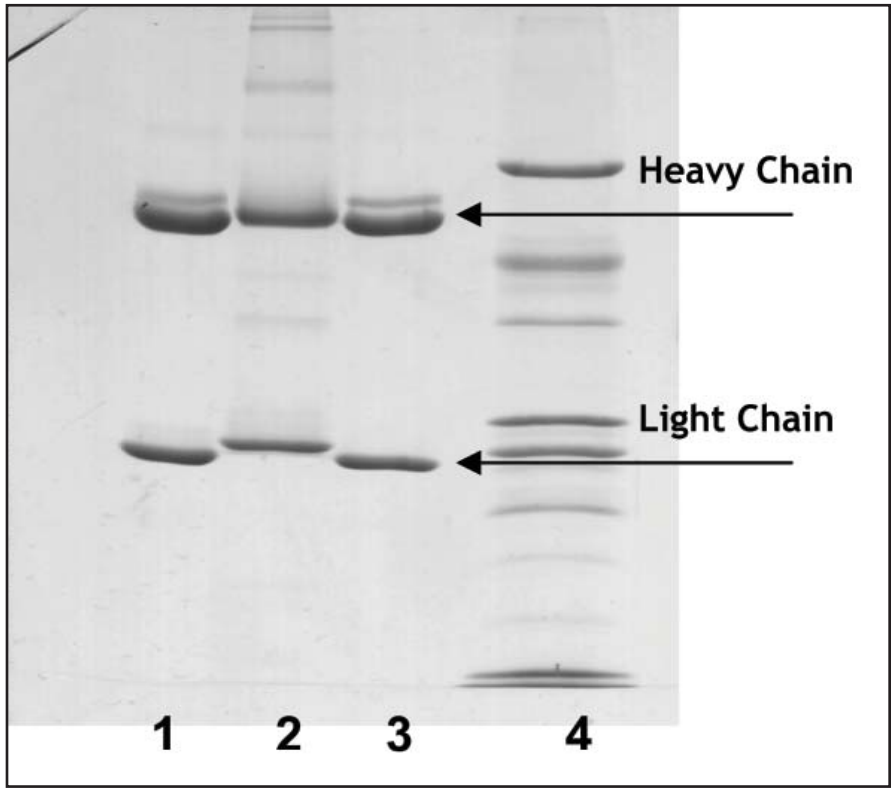

Figure 1. Coomassie blue stained SDS-PAGE of the mAb CB.Hep-1. Lane 1: mAb CB.Hep-1 produced by ascitic fluid. Lane 2: Plantibody HB-01. Lane 3: mAb CB.Hep-1 produced by HFB. Lane 4: Molecular weight marker (BSA, 66 kDa; gliceraldehide 3 phosphate dehydrogenase, 36 kDa; carbonic anhidrase, 29 kDa; chymotrypsinogen, 25 kDa; trypsin inhibitor, 20 kDa; α-lactoalbumin, 14 kDa).

Table 1. Glycan structures of the mAb CB.Hep-1 produced by ascitic fluid, HFB, and transgenic plants.

mAb Source	Structure Nomenclature	Structure Percentage (%)
Ascitic Fluid	$GlcNAc_2\,Man_3(F^6)GlcNAc_2$ $GalGlcNAc_2\,Man_3(F^6)GlcNAc_2$	73.9 21.8
HFB	$GlcNAc_2Man_3(F^6)GlcNAc_2$ $GalGlcNAc_2Man_3(F^6)GlcNAc$	35.7 26.4
Transgenic Plants	$Man_7GlcNAc_2$ $GlcNAcMan_3(X)GlcNAc_2$ $GlcNAc_2(X)Man_3GlcNAc_2$	61.0 5.8 8.2

V-region carbohydrate remains unclear, but a number of studies suggest that its presence may influence affinity and/or specificity.[37-39]

Stevens *et al.*[40] reported an analysis of the mAb stability in transgenic plants that were grown under high and low irradiation at 15°C and 25°C. The conclusion was that plants grown at 25°C developed faster but contained less antibody per amount of leaf tissue than plants grown at 15°C. The antibody content also showed a strong decline during the leaf development, and the heavy chains of mAb underwent degradation via relatively stable fragments.

In vitro incubation of purified plantibody with leaf extracts of wild-type tobacco indicated the involvement of acid proteases at pH 4.0 and 7.0, and the same antibody produced by hybridoma cells exhibited higher stability. They assumed that the plant type N-glycosylation contributed less to the stability of the antibody than the mouse N-glycosylation. Taking into consideration the information contributed by this group, we designed a study to know the stability of the plantibody HB-01 in the clarified tobacco leaf extract, and also to confirm the role of the N-glycosylation on the stability of this antibody.

Figure 1 shows the SDS-PAGE under reduced conditions of mAb CB.Hep-1 produced by ascitic fluid, HFB, and transgenic plants (analytical scale) used in this study to spike the non-transgenic tobacco extract. Sample profiles resulted in two major bands that react with goat polyclonal anti-mouse IgG in immunoblotting assay (data not shown in detail). Obviously, these two bands corresponded with the heavy and light chains of the mAb. The IgG light chain of the mAb CB.Hep-1 produced by the hybridoma cell line (in ascitic fluid and HFB) showed more mobility than the plantibody's light chain. This may be explained by the fact that the plantibody's heavy and light chains contain at least a tetrapeptide (KDEL), added to increase the retention at the endoplasmic reticulum.

This was not due to a light chain degradation of the mAb produced by the hybridoma cell line. Less differences in the heavy chain's mobility was observed, perhaps by a lower resolution of the SDS-PAGE mini-gel in this zone.

Our glycosylation structure analysis demonstrated that glycosylation structures of mAb CB.Hep-1 produced in ascitic fluid and HFB were almost similar. Other structures were also observed in the case of mAb CB.Hep-1 produced by HFB, but in low percentage. With regard to the plantibody HB-01, three predominant main structures were obtained. All contained mainly oligomannose type N-glycans and the (β 1,2) xylose group, but lacked the terminal galactose, sialic-acid residues and the glycans with the plant specific (α 1,3)-fucose (Fuc) (Table 1). Our hypothesis is that these minor differences in the glycan structures could potentially change the stability of the mAb, as compared to the native forms. These carbohydrate group differences could, in theory, change the conformation of the protein and consequently, the accessibility to proteolytic cleavage.

The susceptibility of plantibody HB-01 to proteolytic degradation was compared to the susceptibility of the mAb CB.Hep-1 (produced by ascitic fluid and HFB) by incubating these samples with tobacco leaf extract at different temperatures. The extract was used without the

Table 2. SDS-PAGE of mAb CB.Hep-1 light and heavy chains in tobacco extract at 15°C.

Purity of mAb Light and Heavy Chains	Source of Antibody	0 h	5 h	10 h	15 h	24 h
Heavy Chain (%)	Ascitic Fluid	48.20	47.00	46.33	50.59	44.89
Light Chain (%)	Ascitic Fluid	38.81	37.33	44.53	38.74	44.55
Heavy and Light Chain (%)	*Ascitic Fluid*	*87.11*	*84.31*	*90.88*	*89.33*	*89.41*
Heavy Chain (%)	HFB	36.88	36.10	40.40	34.42	37.07
Light Chain (%)	HFB	39.98	40.40	33.50	36.77	46.84
Heavy and Light Chain (%)	*HFB*	*76.86*	*76.50*	*67.92*	*70.81*	*83.91*
Heavy Chain (%)	Tobacco Plant	49.30	54.19	46.00	50.11	43.79
Light Chain (%)	Tobacco Plant	26.60	20.46	30.21	30.18	28.34
Heavy and Light Chain (%)	*Tobacco Plant*	*75.90*	*74.65*	*76.21*	*80.29*	*72.13*

Table 3. SDS-PAGE of mAb CB.Hep-1 light and heavy chains in tobacco extract at 25°C.

Purity of mAb Light and Heavy Chains	Source of Antibody	0 h	5 h	10 h	15 h	24 h
Heavy Chain (%)	Ascitic Fluid	50.25	45.62	39.73	37.54	38.69
Light Chain (%)	Ascitic Fluid	28.49	34.00	41.28	42.96	33.39
Heavy and Light Chain (%)	*Ascitic Fluid*	*78.74*	*79.62*	*88.01*	*80.50*	*72.08*
Heavy Chain (%)	HFB	40.20	42.19	47.44	43.20	42.49
Light Chain (%)	HFB	29.83	29.70	27.33	28.44	29.36
Heavy and Light Chain (%)	*HFB*	*70.03*	*71.89*	*74.67*	*71.69*	*71.85*
Heavy Chain (%)	Tobacco Plant	42.177	49.30	42.76	41.98	33.31
Light Chain (%)	Tobacco Plant	34.257	23.60	28.45	29.67	30.73
Heavy and Light Chain (%)	*Tobacco Plant*	*76.37*	*72.90*	*71.21*	*71.65*	*64.04*

Table 4. SDS-PAGE of mAb CB.Hep-1 light and heavy chains in tobacco extract at 37°C.

Purity of mAb Light and Heavy Chains	Source of Antibody	0 h	5 h	10 h	15 h	24 h
Heavy Chain (%)	Ascitic Fluid	41.92	43.21	36.25	45.84	30.17
Light Chain (%)	Ascitic Fluid	33.73	33.97	41.87	39.26	42.00
Heavy and Light Chain (%)	*Ascitic Fluid*	*75.65*	*77.18*	*78.12*	*85.10*	*72.37*
Heavy Chain (%)	HFB	51.32	50.38	47.26	49.21	45.89
Light Chain (%)	HFB	32.23	32.49	38.31	33.78	36.43
Heavy and Light Chain (%)	*HFB*	*83.55*	*82.87*	*85.57*	*82.99*	*82.32*
Heavy Chain (%)	Tobacco Plant	44.81	42.04	44.28	46.20	35.63
Light Chain (%)	Tobacco Plant	37.63	38.90	37.63	36.84	33.92
Heavy and Light Chain (%)	*Tobacco Plant*	*82.48*	*80.90*	*81.95*	*83.04*	*69.57*

presence of any exogenous proteolytic activity inhibitor. Results demonstrated a high stability of this mAb at 15°C, 25°C, and 37°C, since no significant differences were observed as compared to the purity of the heavy and light chains of the initial samples (Tables 2, 3, 4 and 5; Figure 2). Therefore, we did not find acid protease degradation at 5.5, and the same antibody with different glycosylation structures exhibits almost the same stability. This is in contradiction with results reported by Stevens *et al.* and Koschuh *et al.*[25,40]

Table 5. Regression analysis for each temperature and source. Samples were considered stable to the tobacco extract conditions and temperature when the slope was not significant: p>0.05.

Source	Temperature (°C)	Intercept	Interval of Trust	*p*
Ascitic Fluid	15	86.280	80.41-92.15	*0.29*
	25	80.878	72.18-89.56	*0.34*
	37	77.412	64.08-90.73	*0.94*
HFB	15	77.412	64.08-90.73	*0.94*
	25	71.338	66.84-75.82	*0.59*
	37	81.928	73.83-90.02	*0.18*
Tobacco Plant	15	76.216	67.82-84.60	*0.85*
	25	76.446	71.13-81.76	*0.62*
	37	84.340	72.28-96.39	*0.22*

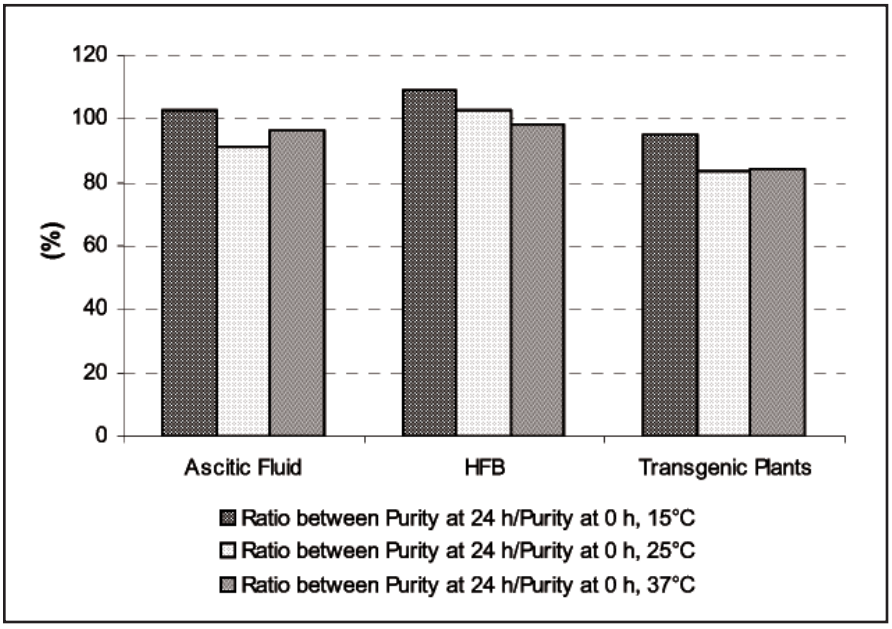

Figure 2. Ratio between the percentage of purity of the mAb CB.Hep-1 at 24 hours and the percentage of purity of the mAb CB.Hep-1 at the initial time of the experiments.

The analysis of the mAb antigen recognition capacity indicated that this parameter was only affected at 37°C for all cases (Figure 3). About 15% decrease of antigen recognition capacity was obtained for mAb produced by the ascitic fluid (10 hours) and for the HFB (5 hours). We considered this decrease irrelevant because 15% falls within the inter-assay variation coefficient of the ELISA used to measure the concentration of the mAb in the leaf extract. In the case of the plantibody HB-01, the assay used to measured antigen recognition capacity has a higher degree of interference than the cases of mAb produced by ascitic fluid and hollow fiber bioreactor. Therefore, we only appreciated about 20% of decrease at 37°C (24 hours).

To corroborate the results obtained in this experiment, the authors purified the plantibody HB-01 from tobacco plant extract at the beginning and at the end of the incubation time (24 hours at 25°C). Figure 4 shows a comparison of the recovery and the SDS-PAGE purity of the plantibody HB-01. Neither parameters showed significant differences. The statistical analysis used to compare these results was the U test of

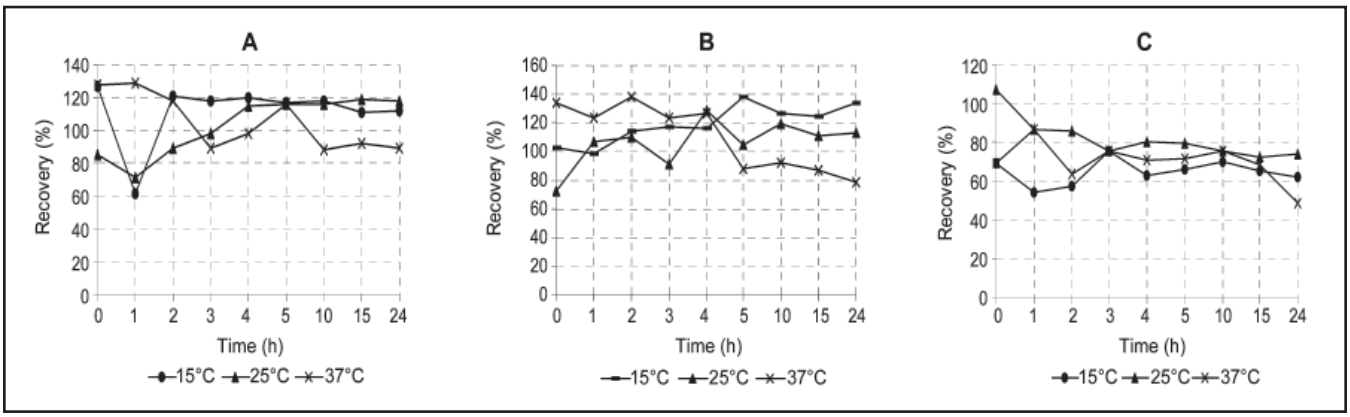

Figure 3. Antigen recognition capacity measured by ELISA of the mAb CB.Hep-1 in tobacco extract at different temperatures during 24 hours. A: mAb CB.Hep.1 produced by ascites method. B: mAb CB.Hep-1 produced by HFB. C: Plantibody HB-01. These data represented on the graphics are the recovery percentage of true values over expected values of mAb concentrations.

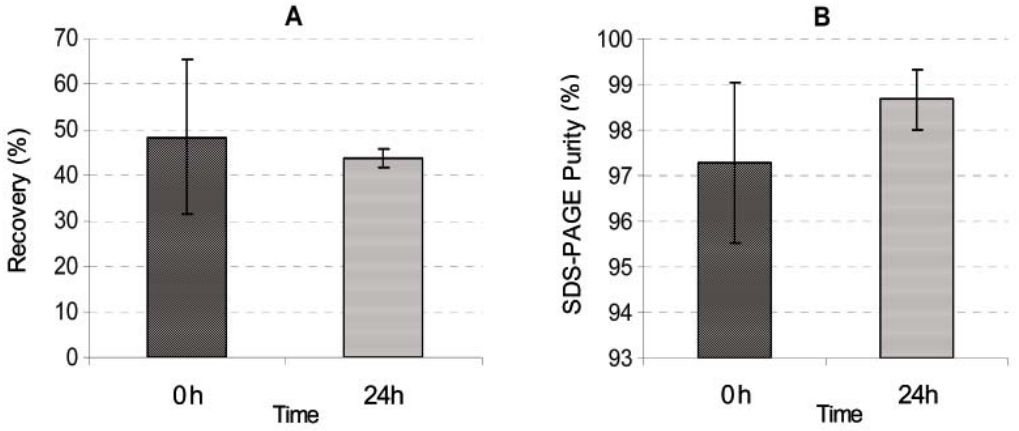

Figure 4. Purity and recovery of the plantibody HB-01 purification before (A) and after (B) its incubation in plant extract at 25°C ($p = 0.662$ for recovery and $p = 0.184$ for SDS-PAGE purity).

Mann-Whitney for a $p > 0.05\%$. The probability calculated for the recovery was $p = 0.662$ while for the SDS-PAGE purity was $p = 0.184$.

Conclusion

These data suggest that no extensive proteolytic degradation of mAb CB.Hep-1 was observed in tobacco leaf extract (pH 5.5 at 15°C, 25°C, and 37°C) in a time period of 24 hours. Differences in the glycosylation pattern did not increase the susceptibility of the mAb CB.Hep-1 molecule to the tobacco leaf extract conditions at 15°C, 25°C, and 37°C in 24 hours. The antigen recognition capacity of the mAb CB.Hep-1 was affected in tobacco leaf extract at 37°C as follows: 15%, mAb produced by ascitic fluid (10 hours); 15%, mAb produced by HFB (5 hours); and 20%, mAb produced by transgenic plants (24 hours).

The contribution of this study is thus: The proteolytic degradation and the loss of the antigen recognition capacity of the plantibody HB-01 in the tobacco leaf extract pH 5.5 does not affect the yield of the purification process; neither does it affect the homogeneity of the molecule, allowing the post-harvest handling and processing of large amounts of this source in a period of 24 hours.

ACKNOWLEDGEMENTS

Authors greatly acknowledge Raudel Sosa, Lena Hernández and Jorge Reconde for the process control support.

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