Generation of a *Nicotiana benthamiana* process-specific host cell protein antibody reagent using a chicken host species

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ABSTRACT

Plants are gaining momentum as an alternative for use in biopharmaceutical manufacture of therapeutic proteins. Some of the more notable species are tobacco, carrot, and thale cress. Tobacco plants (*Nicotiana benthamiana*) are able to produce large quantities of human proteins rapidly and economically in a process commonly referred to as Plant Molecular Farming (PMF). PMF has grown and advanced considerably over the past two decades, with at least 13 companies currently using PMF platforms as well as nine drugs that are presently in National Institutes of Health clinical trials.

As in more commonly used manufacturing host species, recombinant proteins and antibodies produced in PMF platforms also require monitoring of host cell protein (HCP) contaminants using immunoassays and other orthogonal methods. HCP antibody reagent generation specific for plant systems is not as well established as its mammalian or *E. coli* counterparts. As a HCP antibody generation host species, chickens provide a unique opportunity to generate broad coverage HCP specific antibodies. This is due to the lack of homology between chicken and common hostcell models [1].

Here we present the successful use of a chicken host species for the generation of a process-specific HCP antibody reagent to the PMF system. A tobacco in-process sample was used to immunize chicken host species for the generation of antisera and purified IgY antibody reagent. Data are presented that show rapid development of a broad coverage polyclonal antibody reagent in the chickens. The polyclonal antibody was screened by immunoblot and ELISA, with overall coverage being assessed using 2D western blot. This work suggests a strong alternative species to rabbit for the generation of HCP reagents. Further effort to produce a tobacco process-specific immunoassay is underway.

INTRODUCTION

The use of recombinant therapeutic proteins is an increasingly common approach in the treatment of a wide range of human diseases. Biopharmaceuticals employ host organisms such as bacteria, yeast, plants, insect cells and mammalian cells as the manufacturing platform to produce these recombinant therapeutic proteins [2,3]. In the current study, tobacco plant (*Nicotiana benthamiana*) was used for the expression of recombinant vaccine proteins [2,3,4].

Endogenous host cell proteins (HCPs) co-expressed with the therapeutic product during production may constitute a safety issue and must be closely monitored and adequately removed in order to prevent potential adverse effects in patients.

Several analytical methods for detecting and monitoring HCPs in-process and in the final product are available. Well-established immunoassays that use process or platform specific polyclonal antibody reagents as well as mass spectroscopy are some of the methods employed for HCP detection. HCP immunoassays are more cost effective, but rely on the ability of anti-HCP antibodies to successfully detect a broad range of HCP impurities with a high-level of sensitivity. Thus, the performance of the assay is tightly linked to the quality of the antibody reagent used.

Historically, rabbits (most common) and goats (less common) have been used as the host organism for the generation of broad coverage polyclonal antibodies. In the current anti-HCP antibody generation study we focus on the generation of an anti-tobacco HCP antibody reagent. Prior to the present chicken-based study, a rabbit antibody generation system was attempted (data are not reported here). These initial attempts to generate tobacco plant-specific antibodies using rabbit

Hydroponic plant growth systems make tobacco well suited for biopharmaceutical manufacturing.

models failed to generate antibodies with sufficient specific activity in the final qualification. Chicken hosts are becoming increasingly popular as a model for large-scale polyclonal antibody generation. There are several distinct advantages to using chickens over rabbits, or other host animals, for the generation of polyclonal antibodies. Chicken IgY is functionally similar to IgG and therefore can be used in already established immunoassay protocols. The phylogenetic distance of chickens also allows for a more rapid and greater coverage from the immune response to mammalian and other eukaryotic cell lines such as plant. IgY is naturally highly concentrated in the egg yolks of immunized chickens, which can result in extremely high yields of purified antibodies. As IgY is purified from harvested eggs instead of animal sera, the antibody generation process is also more humane and economical.

In this work chickens are immunized following a strategy and schedule similar to those commonly used with mammalian host animals. This method allows for an easy comparison of the speed and strength of the expected immune response. As shown here, the efficacy of these strategies was evaluated by examining coverage in one-dimensional (1D) and two-dimensional (2D) western blotting, as well as, ELISA and slot blot assays[5].

ANTIBODY GENERATION STRATEGY

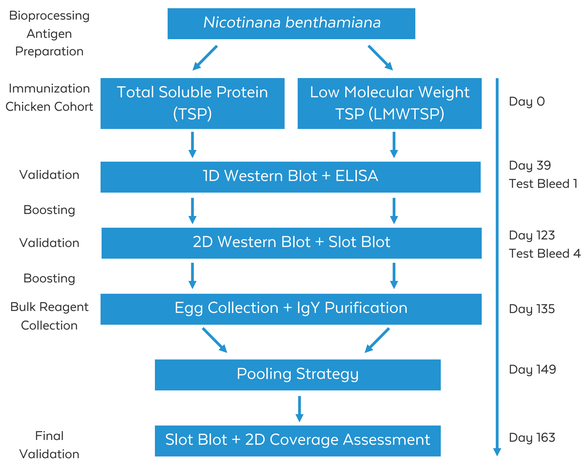

Figure 1 Strategy for generation of an anti host cell protein antibody. A chicken host offers several benefits including: a rapid, robust and broad immune response against cell lysates, and yields of five eggs per week. In this 160 day protocol, four animals yielded 48 eggs, and 5.44 g of purified IgY antibody (equivalent of about 400 mg antibody per animal per week during collection.

Comparison of antibody yields per week from chicken, rabbit, and goat host animals	Chicken	Rabbit	Goat
Animals	5	5	1
Eggs/Week	25	-	-
Antisera/Week (mL)	-	90	350
Ig Yield/Week (g)	2.13	0.63	1.70
Ig yield after 4 weeks (g)	8.5	2.5	7.0
Affinity purified yield (g), 4 weeks	0.43	0.13	0.35

Figure 2 Theoretical comparison of antibody yields per week from chicken, rabbit, and goat host animals. A typical animal cohort would include either 5 or 10 animals for chicken or rabbit, and would utilize from 1-3 goats. A stable long term supply of antibody reagent, about 5 g, can be generated in as little as 160 days from chicken. Rabbits and goat are useful for antibody generation but require almost twice as long to achieve the same antibody yields.

REFERENCES

1. Chicken Antibodies: taking Advantage of Evolution – A Review, Anders Larson et al., *Poultry Science* (1993) v72 pp 1807-1812
2. Plants as Factories for Human Pharmaceuticals: Applications and Challenges, Jian Yao et al., *Int J Mol Sci.* (2015) v16 pp 28549-28565
3. Safety and immunogenicity of a plant-produced recombinant hemagglutinin-based influenza vaccine (HAI-05) derived from A/Indonesia/05/2005 (H5N1) influenza virus, Chichester JA et al., *Viruses* (2012) 4(11):3227-44.
4. Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. Yusibov V et al., *Hum Vaccin.* (2011) (3):313-21
5. A quantitative slot blot assay for host cell protein impurities in recombinant proteins expressed in *E. coli* Zhu D et al. *J Immunol Methods.* (2005) 306(1-2):40-50

ANTIBODY DEVELOPMENT

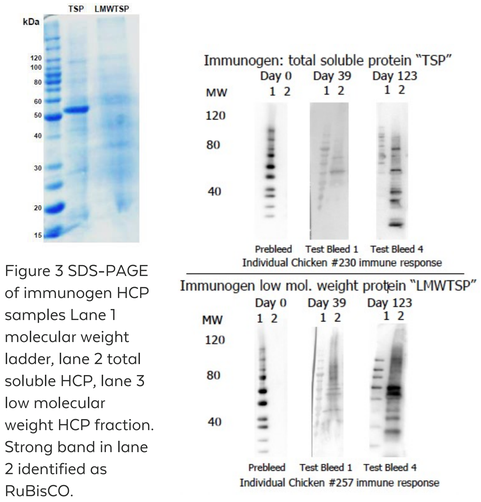

Figure 3 SDS-PAGE of immunogen HCP samples Lane 1 molecular weight ladder, lane 2 total soluble HCP, lane 3 low molecular weight HCP fraction. Strong band in lane 2 identified as RuBisCO.

Figure 4 1D Western blot of representative immunocoverage. Chickens were immunized with either total soluble protein (TSP) or with low mass fraction TSP (LMWTSP) according to a the schedule shown figure 1. Pre bleed sera was taken prior to immunization and blotted against TSP. Test bleed 1 antisera was drawn at day 39, and test bleed 4 antisera was drawn at day 123. Lane 1 molecular weight ladder, lane 2 antisera blotted against TSP.

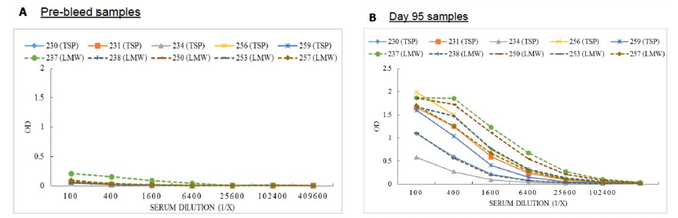

Figure 6 ELISA for (A) pre-immune sera and (B) sera collected at day 95 for each immunogen cohort. Selected representative ELISA are shown for all animals in the study. Solid lines: Samples form TSP immunized animals. Dotted lines: Samples from LMW TSP immunized animals.

Antigen	Ch#	Pre-bleed		Day39		Day67		Day95	
		Anti-TSP	Anti-LMW TSP	Anti-TSP	Anti-LMW TSP	Anti-TSP	Anti-LMW TSP	Anti-TSP	Anti-LMW TSP
TSP	230	25	25	400	400	400	400	100	400
	231	25	25	100	100	400	400	1600	1600
	234	25	25	100	100	100	100	100	100
	256	25	25	6400	1600	1600	1600	1600	1600
	259	25	25	6400	6400	400	400	400	400
LMW TSP	237	1600	1600	1600	1600	400	400	6400	6400
	238	100	25	6400	6400	6400	1600	1600	1600
	250	100	100	25600	1600	400	6400	6400	6400
	253	25	100	100	400	100	400	400	400
	257	25	25	6400	1600	1600	1600	1600	1600

Figure 5 ELISA summary table. Table above shows endpoint ELISA titer data for each immunogen cohort immunized with either TSP or with LMW-TSP respectively. Note chicken 237 is highlighted in peach to indicate a high titer in the pre-immune sera but not excluded from the cohort. Selection of specific host animals was based on titers and coverage in 1D western blots. Host animals 230, 237, 256, and 257 were selected and used for IgY fractionations from yolk. The final IgY superpool was created based on coverage measured in intermediate 2D western blots (not shown).

ANTIBODY EVALUATION & VALIDATION

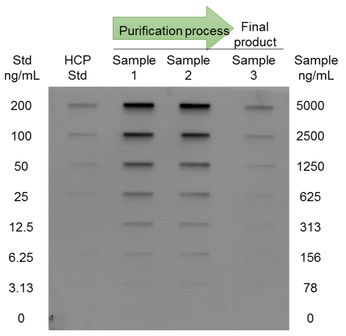

Figure 7 Slot blot analysis of residual host cell proteins (HCP) in in-process samples using anti-plant HCP IgY. Serially diluted plant HCP standard (HCP Std) and samples of a recombinant protein produced in plants were bound to nitrocellulose membrane and incubated with pooled anti-plant HCP IgY. Protein bands were visualized with HRP-conjugated anti-IgY antibody. Sample 1: After the 1st purification step. Sample 2: After the second purification step. Sample 3: Final purified product.

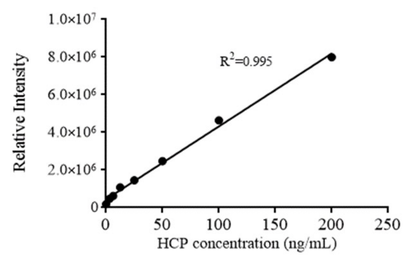

Figure 8 Slot blot assay standard curve. Standard curve was generated using plant HCP recognized by pooled anti-plant HCP IgY. Data shows linearity of dilution.

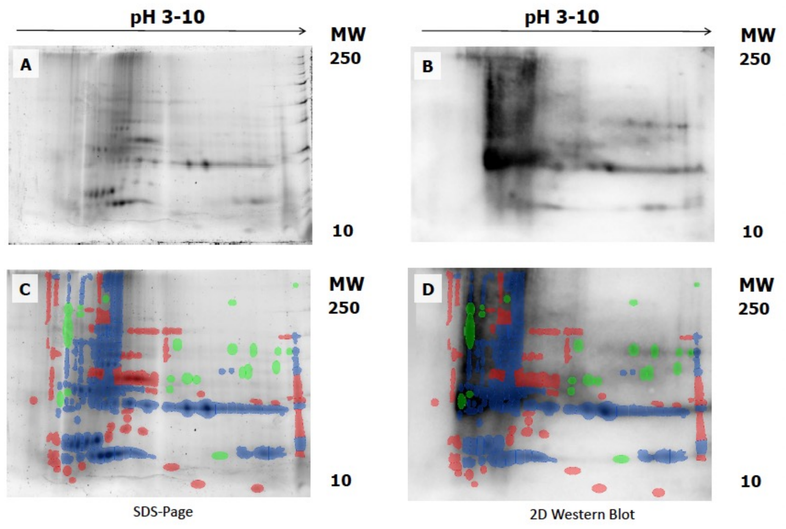

Figure 9 2D Western blot analysis of anti-MT2 HCP antibodies against TSP sample. Total soluble proteins were separated on 2D SDS-PAGE and blotted on PVDF to analyze immunocoverage of the antibody. Antibodies were purified by total IgY fractionation and used to assay for coverage levels. (A,C) Oriole stained protein gel, (B,D) Membranes were probed chicken anti-HCP overnight at 4°C and detected using peroxidase conjugated anti-Chicken secondary antibody at a concentration of 1:10,000 for 30 minutes at ambient temperature. Membranes were incubated using a chemiluminescent substrate and imaged on the BIO-RAD ChemiDocMP™ System using the automatic detection setting. Red indicated spots detected only on the gel, green are spots detected only on the blot membrane, and blue indicates spots detected in both gel and blot.

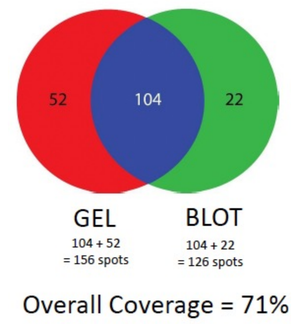

Figure 10 Coverage report for anti-tobacco HCP antibodies. Spots were counted and categorized. Red circle indicates spots detected only on SDS-PAGE, Green circle indicates spots detected by antibody only, and Blue indicates spots detected on SDS-PAGE and in western blot. The coverage is defined as the total number of spots detected on the western blot divided by the total number of detected spots; (126 blot spots)/(178 spots).

CONCLUSION & FUTURE DEVELOPMENT

- Chicken is a suitable host for development of anti-tobacco HCP antibody reagents
- Time to obtain a broad immunocoverage against total soluble protein from tobacco was 95 days.
- Total coverage of 71% was determined using 2D western blot
- Spike recovery assays indicated that the purified tobacco-HCP antibody reagent suggested at linearity of dilution, and recovery percentages in +/- 20 (data not shown)
- The reagents will proceed into final assay development and are intended to be used in future release assays.