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James Stewart New
University of Central Florida

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PLANT-MADE ORAL VACCINES: EVALUATION OF CAPSULES

by

JAMES STEWART NEW

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ABSTRACT

Antigen expression through the Chloroplast Transformation Technology (CTT) produces bioencapsulated subunit-vaccines, capable of eliciting immune responses when delivered orally. Considerable challenges to effective plant-based vaccines are the normalization of dosage and preservation of accumulated antigen, which is complicated by variable high water content and protease activity. This study critically examines the efficacy of lyophilization in dehydrating plant-tissues and preserving plant-derived antigens with vaccine potential.

Lyophilization was optimized through gravimetric analysis using lettuce expressing Protective Antigen (PA) of *Bacillus anthracis (*LS-HPAG) and the human autoantigen Proinsulin (Pins) fused to Cholera toxin subunit B (LS-CTB-Pins). Lyophilization for 48-hours was sufficient treatment to reduce lettuce to 4.57% of its original weight, which retained .058% water content in the bound state; these levels corresponded with oven-dried controls while antigen was stabilized for over a year of storage at room temperature. A simulated gastric fluid assay was applied to evaluate stability of plant derived antigens during digestion. It was observed that lettuce plant cells conferred protection through antigen bioencapsulation for up to an hour under enzymatic digestive conditions.   LS-HPAG immunogenicity was then demonstrated through the induction of a PA-specific IgG response by through oral boosting of C57/BL6 test mice. Survival during toxin challenge demonstrated a protective immune response if 40% of animal immunized by plant-derived PA. Lastly, the inclusion of excipient and adjuvant additives will be considered and utilized for the development of prototype vaccine capsule formulations.

DEDICATION

I would like to dedicate this work to all those people who have supported me through my undergraduate career, including the faculty and administration of the University of Central Florida, the Staff members of the Biomedical Sciences Department and the other members of the Daniell Lab. Furthermore, I would like to dedicate this to my parents and family who have kept me enthusiastic and excited about my wonderful undergraduate research experience at UCF, and motivated me to continue my drive to accomplish significant biomedical research.

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ABBREVIATIONS

Atx: Anthrax toxin

AVA: Anthrax Vaccine Adsorbed

AVP: Anthrax Vaccine Precipitated

CD: Cluster of differentiation

DCs: Dendritic Cells

CTT: Chloroplast Transformation Technology

CTB: Cholera Toxin B Subunit

EF: Edema Factor

ELISA: Enzyme Linked Immunosorbent Assay

FD: Freeze-dried

FW: Fresh Weight

GM-1: Monosialotetrahexosylganglioside

IFN-γ: Interferon Gamma

IgG: Immunoglobulin G

IL-2R: Interleukin 2 receptor

LF: Lethal Factors

LS-CTBPins: Lactuca sativa expressing Proinsulin fused to CTB

LS-HPAG: Lactuca sativa expressing His-tagged Protective Antigen

NOD: Non-obese diabetic

PA: Protective Antigen

RWC: Relative Water Content

SDS-PAGE: Sodium dodecyl sulfate Polyacrylamide gel electrophoresis.

T1DM: Type 1 Diabetes Mellitus

TGF-β: Tumor Growth Factor β

Ti: Tumor infecting plasmid

TP: Transgenic Protein

TSP: Total Soluble Protein

VLP: Virus Like Particle

INTRODUCTION

Since the FDA approval of recombinant human insulin in 1982, recombinant biopharmaceuticals have exploded into the market. Approaches utilizing the translational machinery of diverse cell types, involving both stable and transient expression systems have successfully produced biologically active proteins. Among the various biopharmaceuticals produced are vaccine antigens, monoclonal antibodies, and antimicrobials. Despite the availability of these technologies, high production costs occlude much of the population from receiving potential treatments. These high costs stem primarily from downstream processing which often contributes 80% of total production costs. In light of this issue, plants offer an appealing alternative. As self-sustaining bioreactors, all energy is derived from the sun. In addition, purification is often unnecessary due to annulled risk of pathogen contamination.

The transformation of a plant cell can occur through the modulation of either of two genomes: the nuclear and the chloroplast genomes [1]. Although the younger of the two, Chloroplast Transformation Technology (CTT) holds significant promise in the realm of bioengineering [2, 3]. A technology that owes its origins to the reintroduction of transformed organelles to protoplast cell lines [4], chloroplast transformation now occurs through particle delivery system of expression vectors facilitated by gold or tungsten microparticles [5, 6]. Vectors initiate stable integration into the chloroplast genome via homologous recombination within a targeted flanking region [7]. Integration is therefore site-specific, and expression is normalized across homoplasmic plant lines, which have accomplished unheralded levels of protein expression as high as 72% of the plant's total

soluble protein (TSP) [8, 9]. The maternal nature of the chloroplast provides a form of inherent gene containment, and its plasmid nature renders it free of gene silencing mechanisms and position effects, while enabling polycistronic processing [2, 8-11]. In addition to transcriptional advantages provided by chloroplast based expression, translation of mRNA transcripts results in disulfide bond formation [12], lipidation, and proper protein folding facilitated by chaperonins [13]. These properties make chloroplasts ideal sites of synthesis for recombinant proteins.

The expression of vaccine antigens in the chloroplast was first conceived in 2001 with the expression of Cholera Toxin Subunit B in tobacco chloroplasts [14]. Since this implementation the capacity of antigen expression has included immunogens with bacterial, viral, plasmodial[15], and human origins[16]. Additionally, chloroplasts have proven their capability to form highly immunogenic, complex antigen superstructures such as virus-like-particles (VLPs) and capsomeres [17-21]. The accumulated antigen is protected from digestion by the plant cell walls, enabling oral delivery of vaccine antigen to target the gut mucosa [22]. Within the intestinal epithelium, resident plasmacytoid dendritic cells of the lamina propria, as well as microfold epithelium cells, will aid in the sampling of antigen as it passes through the gastrointestinal tract. Induction of immunity against the vaccine antigen will occur via dendritic cell presentation to Th1 and Th2 lymphocytes, prompting inflammatory responses and activation of antibody producing B cells in both the systemic and mucosal levels of immunity [23].

Despite the potential of this platform for oral vaccine production, the variable expression of recombinant antigen resulting from differing water content, leaf age, and harvest time of day limits batch consistency. Furthermore, the cold-chain transport

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required to maintain vaccine viability contributes to growing costs and hindrances to distribution. These issues are highlighted by the lack of plant-derived therapeutics from the current pharmaceutical market. An effective means of plant-derived vaccine preservation is therefore needed to make this technology practical for human use.

Lyophilization, or freeze-drying, is a common practice of the food and herbal industries because it preserves the aesthetic appeal of plant materials and is also used by many industries to dehydrate and concentrate certain solutions, such as purified proteins. The effects of lyophilization on accumulated vaccine antigen were observed, as transplastomic materials were dried. The efficacy of such freeze-dried vaccine antigens was then examined with respect to antigen viability, gastric stability, and immunogenicity in an effort to evaluate the efficacy of plant-mediated oral immunization.

BACKGROUND

Chloroplast Transformation Technology

The dawn of plant genetic engineering owes itself to the discovery of a unique bacterium, Agrobacterium tumefaciens, the causative agent of the crown gall disease, or plant tumors. A. tumefaciens utilizes a plasmid with amazing functions to infect wounded plants in order to produce an ecological niche for itself and its progeny. Named by the scientists that discovered it as the Tumor inducing (Ti) plasmid, this plasmid contains a special segment of DNA that dissociates from the Ti plasmid upon plant-cell conjugation and autonomously inserts itself into the plant's genomic DNA [24, 25]. This modification serves several functions, which include the induction of several plant hormones, auxins and cytokinins, that promote cellular proliferation and give rise to the ensuing tumor. The T-DNA further diverts the endogenous metabolic pathways to favor the synthesis of unique amino acid derivatives that only A. tumefaciens can utilize as a source of nitrogen, known as Opines [25]. This elegant system for manipulating host DNA quickly became an attractive platform for use in bioengineering plants with improved agricultural traits.

Eliminating the virulence genes from this plasmid and replacing them with any gene of particular interest soon became a common technique for engineering transgenic plants. Later, the production of binary vector systems enabled researchers to work seamlessly with the same plasmid from *Escherichia coli*, to *A. tumefaciens*, facilitating ease in this technique. However, widespread use of this system demonstrated the pitfalls of this transfection method. Firstly, the site of gene integration is random. Secondly there is no regulation over the number of integration events, resulting in widely variable gene copy numbers in different plant lines. These events compound with each other to give rise to a

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number of genetic occurrences that further confound the expression of a target gene. The position of integration produces expression variability through position effects, which influence the frequencies that certain chromosomal areas are transcriptionally accessible, through manipulation of chromatin density. Plant cells, which by eurkaryotic nature contain machinery necessary for regulating the chromosome complexes in cells, therefore threaten gene-of-interest expression with gene silencing mechanisms.

This lack in control over gene insertion could possibly result in phenotype altering pleiotropic effects that may manifest if the site of integration interrupts important endogenous gene sequences.

The drawbacks associated with the Agrobacterium approach to genetically modifying plants prompted researchers to investigate other methods of transformation. Some set their eyes on the chloroplasts of plant cells, the so-called energy producer of the cell. Beyond this organelle's high-energy flux, many of the features of its genome made it a very promising candidate for biotechnology applications. Indeed, most of the pitfalls associated with nuclear genome transformants were overcome by moving the site of gene integration to the descendant of prokaryotic cyannobacteria, the chloroplast [26].

This subcellular organelle is similar to the mitochondria in the sense that it contains its own genome inherited from its prokaryotic ancestors. The chloroplast genome is therefore analogous to that of a prokaryote; it is plasmid, or circular, in nature, and the only structural organization that it is prone to is natural coiling by gyrases which is readily reversed by topoisomerase activity. A lack of chromosomal structure translates into a lack of gene silencing potential, which bodes well for the expression of a transgene. In addition to being a highly polyploidy organelle, the chloroplast exists at high numbers in plant cells.

Each plant cell may have as many as 10,000 copies of the chloroplast genome, which offers a tremendous potential for foreign gene expression [3].

The chloroplast however was not a target of Agrobacterium; therefore, a technique was required that would allow integration of the gene into the genome of the chloroplast. CTT initially began through laborious techniques that involved purifying live and intact chloroplasts, genetically modifying them in a technique akin to bacterial transformation, and then reintroducing those transformed chloroplasts into a plant cell, followed by the subsequent regeneration of those plants [4]. Later, the development of Biolistic Particle Delivery, or gene bombardment methods, greatly reduced the required labor for producing transplastomic, or plastid-transformed, plants [5, 6]. Since its birth, gene bombardment has matured into an optimized technique largely pioneered by the Daniell Lab, which generated the universal chloroplast transformation vector pLD that shuttles target genes into the chloroplast, and initiates a recombination even to transpose the target gene into the host genome [7].

This recombination event is driven by two sequences flanking the target gene that are homologous to sequences in the chloroplast genome. Sequence homology initiates homologous recombination, which positions the gene of interest into the chloroplast plasmid. This technique allows for site specific integration based on the choice of flanking sequence, and the integration is guaranteed to happen only once per plasmid. In addition to the gene of interest, the pLD facilitates the integration of a complete expression cassette: this includes promoters and selectable markers, the hallmarks of any gene vector. After bombardment, transformed cells undergo a non-lethal selection on spectinomycin containing media [27]. Specintomycin is an aminoglycoside which inhibits protein

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synthesis by ribosomal inhibition and therefore halts cellular proliferation [28]. Only cells coengineered with the gene of interest will contain the spectinomycin resistance gene, aadA. Therefore only transformants will be able to grow. Totipotent plant cells eventually regenerate to a full plant and seeds may be collected with one hundred percent of progeny inheriting the transgene [13]. As opposed to nuclear transformed plants that experience mendalian inheritance of transgenes, chloroplasts genes are almost entirely maternally inherited. This unique feature also doubles as an inherent gene containment system, as there is no threat of pollen containing transgenes [10].

The capacity of the chloroplast to express recombinant proteins has been demonstrated through the sheer diversity of publications that have come out of the CTT field [1]. Initial interests were geared towards improving the agricultural viability of many plant species. Genes conferring herbicide resistance, insecticide resistance, and pathogen resistance were soon being introduced to plants [29, 30]; these were followed by such traits as draught tolerance and salt tolerance in carrot [31]. Plants were rendered with phytoremediation capabilities to remove organomercurial compounds that normally inhibit photosynthesis in chloroplasts [32]. The potential of chloroplast biotechnology was eventually tested in the field biopharmaceuticals. The list of accomplishments to date is considerable, ranging from human blood proteins such as Erythropoietin [33] or IFN-γ [12], complex monoclonal antibodies [34], even biopolymer plastics with medical applications [11]. This platform has proven worthy of considerable attention, as it has obtained levels of expression at biomasses unheralded by other systems in terms of cost; plants have expressed as much as 70% of their total protein in the form of the transgene, which translates into enormous yield of transgenic proteins [9]. Now, this platform has

become of interest in the context of vaccine antigen production and has been repeatedly

used in the modulation of murine immune responses *in vivo*.

More commonly known as Anthrax, Bacillus anthracis is in fact a relatively common spore-forming, gram-positive inhabitant of the soil, classified as a Category A agent by the Centers for Disease Control [35]. Natural human infections occur rarely in developed areas due to a lessened risk of exposure and occur at a slightly higher frequency in developing areas; this pathogen's primary hosts are large herbivorous mammals [36]. Human infection typically occurs through the inhalation sporulated *B. antracis* and is often a result of exposure to contaminated animal products but will ultimately result in fatal septicemia [36]. In today's world however, anthrax spores have become a major subject of bioterrorism concerns as weaponized spores designed to optimize their airborne dissemination have been developed. Strikes made against United States citizens by mailing of anthrax spores through the United States Post Office in 2001 demonstrates the need for effective immunization strategies applicable to the general public, and not just members of the armed forces, which prompted major research in the characterization of B. anthracis and its pathogenesis for development of more efficacious second generation immunization strategies [37, 38]. Virulent B. anthracis possess extra chromosomal plasmids that bestow them with their virulent traits. The pX01 plasmid encodes the anthrax toxins protective antigen (PA), edema factor (EF), and lethal factor (LF) encoded by the genes *pagA*, *cya,* and *lef* respectively. The second plasmid, pX02, contains the genes that produce a poly-D-glutamic acid capsule [37].

The inhalation of anthrax spores, leads to their phagocytosis by resident lung alveolar macrophages, as well as migrating neutrophils and dendritic cells and the progression of the early intracellular phase of infection [39, 40]. While the bactericidal

activity of the macrophages and neutrophils may effectively control bacterial proliferation, the success of the endosomal conditions depends largely on the magnitude of bacterial infection. Dendritic cells, however, are believed to favor spore germination and consequently transport invading pathogens to the draining lymnodes of the lungs facilitating their systemic diffusion. Once phagocytosed, the inactive spores begin germinating and mature to the vegetative bacillus state [40]. Evasion of the mechanisms of the phagolysozome by Bacillus anthracis is dependant on an array of defenses against APC phagocytosis including secreted proteases, membrane proteins geared towards neutralizing bactericidal mechanisms and optimizing nutrient uptake. Furthermore, expression of diverse phospholipases maintains a favorable environment by inhibiting endosomal maturation and promoting cell lysis that leads to the release of proliferating cells and the onset of a systemic infection. This release of the active vegetative bacterium into the blood stream characterizes phase II or the systemic anthrax infection [35].

In circulation, Bacillus anthracis begins secretion of the anthrax toxin. Anthrax toxin (Atx) is a three component protein-translocase system designed to modify cellular activity through the modification of cytoplasmic molecules and promote the eventual lysis of the host leukocytes and overall immune dysfunction [39, 41]. Protective Antigen (PA) forms homoheptameric structures as well as oligomers with the other toxin components Edema Factor (EF) and Lethal Factor (LF). Upon secretion Atx targets a variety of endogenous receptors that function as the Anthrax toxin receptor and facilitate the endocytosis of this pathogenic complex. Internalization initiates toxin activation, as the PA subunits undergo furin-based proteolytic clevage, as well as pH-dependant conformational activation before the heptameric PA structure enters a ring-like conformation and internalizes with the

membrane of the endosome to form a trans-membrane pore at which point EF and LF are secreted into the cytosol [40]. EF possesses adenyl cyclase activity and impairs host defenses, particularly phagocytosis, while LF is a zinc dependant metalloprotease that inhibits mitogen-activated protein kinase kinase (MAPKK) and results in cellular lysis [40]. Systemic proliferation of this bacterium promotes further pathogensis of other cell types as multiple other receptors for PA have been identified such as capillary morphogenesis protein 2 (CMG2) or tumor endothelial cell marker 8 (TEM8) [38].

As the spearhead facilitating systemic B. anthracis infection, the Atx system became the target of immunization strategies against anthrax, as its inhibition would effectively neutralize the major virulence factors and halts the proliferation and anthrax [35, 36]. Currently, there are two commercially available anthrax vaccines that are produced through similar approaches: the Anthrax Vaccine Adsorbed (AVA) in the United States and the Anthrax Vaccine Precipitated (AVP) in Europe [36]. AVA, produced by BioPort Corporation of Michigan in 1970 under the trade name BioThrax, induces protective B-cell mediated immunity within three to six doses. AVA is an acellular preparation of B. anthracis cultures prepared through culture filtration and adsorbed onto an aluminum resin, containing Atx as the "subunit" through which the protective response is conferred. EF and LF components, however, result in severe reactogenicity in 20% of patients characterized by erythema, soreness, and fever, with serious adverse effects in 1% of patients [36]. AVP, licensed through the Center for Applied Microbiology and Research of Great Britain in 1979, contains both only PA and LF. AVP requires several primovaccinations followed by a six-month boost of antigen and apparently delivers milder side effects. The severe reactions experienced y recipients of this vaccination highlight the

need for the development of a safer vaccine for the general public. In the case of a bioterrorist threat, the reactogenicity of AVA would occlude safe and proper immunization of certain members of society such as the young, and elderly. The safest measure to the preparation of a subunit vaccine is recombinant DNA technology. In fact, a recombinant version of AVA is already under clinical trials by the Food and Drug Administration, as a pathogen free culture system to produce non-virulent antigens would represent the safest approach to efficacious immunization. Though some criticize the approach of immunizing against a single subunit representative of the pathogen, even including such component as EF and LF severely limits the safety of vaccines against anthrax. Therefore, a next generation vaccine strategy that is optimized for safe yet efficacious immunization is required.

The bioterroist threat associated with anthrax warrants the need for immunization approaches suitable for widespread use by the general public. The severe reactogenicity and relative unavailability of Anthrax Vaccine Adsorbed (AVA), as well as the limited supplies of Protective Antigen (PA), mandate the development of a better vaccine [35, 36]. In 2003, Jennifer Watson et al. of the Daniell Lab published their integration of the PA gene *pag* with tobacco chloroplast [42]. Expression was driven by a light-dependant upstream regulatory element *psbA*, and expression levels of 2.5 mg PA per gram of fresh leaf, or 14% TSP, were achieved under continuous lighting conditions. Calculations provided an extrapolated value of ~400 million vaccine doses per acre of crop. The bioactivity of this antigen was demonstrated through a macrophage lysis assay, wherein the synergism between plant-derived PA and anthrax derived LF killed host cells was retained despite the plastid-source of PA [42].

In a subsequent study, Koya et al. evaluated the efficacy of this tobacco-derived vaccine against anthrax. Using a mouse immunization model, purified plant-derived PA preparation and *B. anthracis* derived PA were delivered in adjuvant subcutaneously, and the induced immune response was measured. IgG titers of 1:320,000 were detected in both test groups [43]. This serum was then subjected to toxin neutralization assay, where it exhibited the capacity to inhibit lethal toxin formation from PA and LF. When exposed to lethal doses of anthrax toxin, mice immunized with chloroplast derived PA in adjuvant exhibited 100% survival rate for 200 hours post challenge.

This study demonstrates the potential of derived PA from transgenic chloroplasts. While the final product is immunogenic, production is not costly and large amounts can be farmed to produce enumerable doses. The latest study involving PA from the Daniell Lab was the expression of this vaccine antigen in lettuce, which achieved expression of 22% TSP [9]. While no evaluation of functionality was completed on this antigen, previous demonstrations of plant derived PA demonstrate its bioactivity, and expressing such vaccine antigens through edible crops could allow for oral administration of antigens and subsequent immunization through the intestinal mucosa. Such orally vaccines would be ideal in bioterrorism contexts, with respect to their ease of administration and minimal downstream processing measures.

Type-1 Diabetes and Recombinant Orally Deliverable Insulin

Diabetes mellitus is a group of diseases characterized by a decrease in pancreatic beta-cell activity which is accompanied by hyperglycemia, or high blood sugar, and in severe cases may lead to such complications as heart disease, kidney failure, ketoacidosis, and stroke [44, 45]. Over 23 million Americans live with this disease, yet research has yet to produce a cure. In 2007, Diabetes was the seventh leading cause of death, and in that year healthcare costs totaled $174 billion in the United States alone. Most importantly, is the cost of currently used recombinant insulin to patients, which might cost as much as $100 a month [46].

In most cases, Diabetes is a result of aberrant insulin signaling resulting in the deregulation of homeostasis. Type-1 Diabetes mellitus (T1DM) results when endogenous insulin production is insufficient, or when the produced insulin is a mutated and inactive form [47]. Insulin is the key hormone which stimulates the uptake of sugar from the blood and its storage as glycogen. Malfunctioning insulin signaling therefore results in an increase in basal blood glucose levels, and also in blood sugar spikes after meals, as they are unable to cope with the sudden increase of sugar from their diet [47]. T1DM must therefore carefully screen what they eat, as well as monitor their daily levels of blood glucose. Patients with T1DM are frequently insulin dependant, meaning that they cannot rely on endogenous and must therefore supplement themselves with other forms of insulin. This process of daily finger-pricking for blood glucose measurements, followed by self-injection of purified insulin, highlights the need for more comfortable means of disease management for those afflicted with T1DM.

In some cases, the onset of Type-1 diabetes is the consequence of an autoimmune

response against the pancreatic beta-cells [44, 48, 49]. A few rare monogenic alleles, as well as several susceptibility loci, have been implicated in this disease [45]. While the major susceptibility locus identified is within the HLA genes encoding for host Major Histocompatability Complex (MHC) proteins, other identified loci include the insulin gene itself, Cytotoxic T-lymphocyte Associated Protein 4 (CTLA4), and Interleukin 2 receptor (IL2R). The involvements of the above loci suggest that this disease is driven by a breakdown in signaling efficacy of the immune response. Research has also strongly suggested an involvement in viral infection during the initiation of the T1D disease state, yet no direct link has yet been identified [47]. These susceptibility loci are often triggered by environmental stimuli with many identified triggers of this autoimmune reaction. Research has shown that certain dietary components could trigger the disease in predisposed test mice; cow milk, particularly its albumin component, promotes β-cell autoreactivty [45]. Another food antigen which initiates the auto reactive response is the wheat protein gluten, though the effect was less than that observed by the cow milk.

Regardless of the sources of initiation of the various immune responses that collectively characterize T1DM, the early phase of this pathology occurs largely unnoticed, producing minimal symptoms. Bone marrow-derived lymphocytes, or B-cells, drive an antibody response, which diffuses through the pancreas, opsonizing cognate autogantigens [47, 49]. As the disease state progresses, however, CD8+ cytotoxic lymphocytes supplemented by the cytokine meileu of CD4+ Helpter T-cells, infiltrate the organ and mediate the destruction of the β-cells [48], which in turn eliminates the metabolic capacity to regulate blood sugar. Several antigens have been identified as the key players in the autoimmunity of T1DM: insulinoma-associated antigen-2 (I-A2), insulin (mIAA), glutamic

acid decarboxylase 65 (GAD65), and zinc transporter 8 (ZnT8) [49]. The presence of several alternative autoantigens as the root cause of this disease complicates the matter in such that therapies must therefore be customized on a patient level.

Insulin remains the most effective drug against both forms of Diabetes in today's market; however, management of this disease through insulin therapy is a tedious process as patients literally walk the thin line between hyperglycemia and hyperinsulinaemia [47]. Compounding this difficulty is the fact that insulin is generally prescribed late in the disease onset and that patient compliance is low. There is therefore a strong push to develop new methods of insulin therapy, and oral delivery systems have become very popular among pharmaceutical companies [50, 51]. Insulin has shown to possess therapeutic potential in patients of both forms of diabetes mellitus. In T2DM patients this therapy serves only to replace endogenous insulin, particularly the quick response insulin response that many T2DMs lack in response to feeding. In T1DM patients, however, the effects of insulin therapy further mediate an immunomodulatory response due to the autoimmune nature of this disease [50, 51]. This mechanism, known as oral tolerance, is becoming widely studied but is largely a dose dependant mechanism. Small antigen doses drive a regulatory FoxP3+ T-cell response, whereas large antigen doses drive clonal deletion and anergy of cognate T-cells; though these exist as disparate responses, research has shown that the two cell-types are actually related and can readily interconvert if given the correct microenvironments of TGF-β [52]. In either case, modifying the cellular actives of insulin-activated T-cells through oral administration of insulin is a lucrative concept. Several pharmaceutical companies have oral insulin products currently in clinical trials, including capsule (Intesulin, Coremed), tablet (Eligen, Emisphere Technologies), and liquid

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forms (HIM-2, Nobex Technologies) [50]. These approaches generally utilize micro- or nanaparticles made of such polymers as alginate, cyanoacrylate, and β-cyclodextrin. They are often further optimized for human delivery through pegylation, protease inhibitors, and pH responsive delivery systems. Oral delivery of insulin is likely to be the new phase of Diabetes disease managements and will replace the discomforting habits of finger-pricking and self-injection.

The first reported expression of a potential therapeutic treatment for diabetes through a transgenic chloroplast approach came from the lab of Henry Daniell in 2007 [16]. Ruhlman et al. achieved stable homoplasmic integration of the human proinsulin gene within lettuce and tobacco chloroplasts through pLD transformation meditated by particle bombardment. Proinsulin (Pins) was fused to the Cholera Toxin B Subunit, which had previously demonstrated oral adjuvant properties, through intertactions with GM-1 receptors in the gut [14]. The construct was expressed as the fusion protein (CTB-Pins), and therefore possessed bivalent properties. In tobacco, CTB-Pins was placed under control of the endogenous, light regulated promoter psbA. Expression levels were unprecendtedly high and were reported to reach as high as 72% [9, 16]. In lettuce, the expression cassette utilized the bacteriophage T7 gene10 promoter and accordingly achieved lower expression levels of around ~24-25% [9].

Ruhlman et. al then evaluated the therapeutic potential for this plant-derived human protein in a non-obese diabetic (NOD) mouse model which has onset of Diabetes after twelve weeks. Using five-week old NOD mice, plant derived CTB-Pins was administered orally in weekly doses for seven weeks. CTB-GFP, IFN-GFP constructs and wild-type leaf material were gavaged to other control groups. After treatment, mice treated

with the plant-derived Proinsulin construct had reduced lymphocytic infiltration of pancreatic tissues; β-cells particularly were preserved when compared with negative control treated mice. Furthermore, mice had lower blood and urine glucose levels, suggesting a return of function to the pancreatic islets. Detection interleukin-4 and interlukin-10 in the pancreas of CTB-Insulin treated mice suggests that the reduced insulitis score was the product of T-helper 2 lymphocyte induction of tolerance, through a subset of T-cell known as Regulatory T-cells (T_{Reg}).

This proof of concept caught on, and others began to replicate this therapeutic model. Another Canadian group published plastid based expression of autoantigen human glutamic acid decarboxylase (hGAD65) through the eukaryotic alga Chlamydomonas reinhardtii during the next year [53]. Further research by the Daniell Lab produced a CTB-Pins construct with three inherent furin cleavage sites (CTB-PFx3) [46]. Upon endocytosis, this therapeutic would be cleaved by endogenous pro-protein convertases, which would process Proinsulin to active Insulin by cleave of the C-peptide. This platform was also shown to reduce blood glucose levels, while the simultaneously delivered C-peptide is said to confer protection from diabetic complications by stimulating nerve and renal functions.

Plant-derived insulin is a very appealing concept: while oral insulin is about to make its debut in the pharmaceutical market, plants possess inherent capacity to resist gastric digestion, thus making them suitable vehicles for delivery of antigen to the intestinal mucosa. Furthermore, chloroplasts are completely capable of forming bioactive insulin through the correct formation of disulfide bonds, which therefore represents an inexpensive production platform. The innate characteristics of plant tissues, however, occlude these plant derived therapeutics reasonable usage: considerable variability in

expression levels, high water content, and protease activity produce widely variable antigen yields. Furthermore, their short half-life under refrigerated conditions renders them unsuitable for storage of an accumulated therapeutic. A method of batch standardization, as well as long-term preservation for storage is therefore needed before plant-derived insulin may be considered a relevant alternative to injectable forms so widely used today.

Lyophilization

Despite the increasing potential of plant-derived antigen-based vaccines, researchers must consider several drawbacks associated with using plants as a vaccine vehicle. Due to the high and varying water content of many edible plant materials, delivering accurate doses is quite challenging. In addition, plants contain unusually high concentration of proteases and secondary metabolites that will interfere with the preservation of the transgenic antigen post harvesting. Thus, the shelf life of transgenic protein (TP) in harvested leaves is usually short unless frozen. Considering these shortcomings, it necessary to devise a method of plant preservation that serves to maintain the therapeutic protein so that the many advantages of plastid based expression may be utilized.

It stands to reason that the removal of water from these plant tissues may mollify the problems associated with plant-based vaccines. In the absence of water, antigen variability would normalize and proteolysis would be inhibited. Lyophilization, commonly referred to as freeze-drying, is the removal of water from frozen tissue through sublimation. In recent years, lyophilization has emerged has a promising means of plant preservation [54, 55]. This procedure, which involves the freezing of the plant in a vacuum and the slight addition of heat to sublimate all water directly out of the cells, serves to effectively preserve the cellular protein content and even the appearance of the material. Though Benedict and Manning first invented the lyophilizer in 1905, it was not until the 1970s that pharmaceutical companies and taxidermists saw the applications of this procedure [55]. It is now widely used by biotechnology companies to produce preparations of purified proteins for biomedical research.

Today, lyophilization is widely used in preparing herbal supplements, foodstuffs, pharmaceuticals, and biotechnology products. A review of this process given by Dr. Dumitru Mnerie of the Polytechnica University of Romania, describes the pros and cons of lyophlization [55]. While most biological processes are stopped in the frozen form, the ice further promotes the maintenance of form and structure, contributing to the appealing appearance of lyophilized products. However, freeze-drying is among the most costly of drying methods, and possible alterations could occur to target molecules changing its physical structure or chemical makeup. Mnerie notes that with high water content foodstuffs such as lettuce, lyophilization is generally less efficient due to the high porosity of the tissues [55].

Mnerie implicates the initial freezing step as critical to the success of the lyophilization protocol in preservation. Even and consistent ice-crystal formation provides a more appealing product. He continues to describe how the duration of this process relies on two inherent factors of the tissue being dried: its conductivity, or how well it transmits temperature and pressures, as well as its permeability. Lyophilization must therefore be optimized for new materials. Sublimation occurs via minute temperature differences between the sample environment and the condensing chamber. This reaction is driven by the continuous reduction of vapor pressure in the vicinity of the sample, because the vacuum is driving all vapors to the condenser where it is subsequently frozen. Drying may essentially be broken into two processes; in the primary drying process the bulk of the water content will be removed leaving approximately 5-7% residual water content. This event may occur quite rapidly, but these levels of water are likely not appropriate for long-term storage. Therefore, a secondary drying process must be allowed to occur which may

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take considerably longer, but will help to reduce residual water content to a range of 1-3%, acceptable levels of moisture for long term storage.

Many groups have already begun utilizing lyophilization measures within their studies in one form or another. The first report of using lyophilization comes from the lab of Amanda Walmsley in Milan, Italy. In 2003 Walmsley et al. published an article wherein they applied lyophilized to nuclear-transformed *Arabidopsis thaliana* leaves to stabilize a E. coli heat-liable Toxin B Subunit-ESAT-6 fusion antigen [56]. In this study, however, lyophilization was applied mainly as a means to concentrate antigen content, as is the intent of many pharmaceutical applications of this process. This group's apparent lack of interest in the preservative properties that lyophilization conferred to their antigen is demonstrated by the fact that the lyophilized plant material was stored consistently at -20°C. However, Walmsley noted the increased level of antigen dose, as well as 4-month stability at -20°C; an interesting demonstration was the GM-1 binding assay with 4-month old samples in comparison to fresh samples. Lyophilization continued to be applied in more instances: Mason et al. stabilized hepatitis B surface antigen (HBsAg) and Norwalk virus capsid protein (NVCP) in transgenic tomatoes and potatoes through lyophilization noting an increased immunogenicity of the virus-like particles when delivered orally to mice [17, 20]. Webster et al. stabilized measles virus hemagglutinin (MV-H) derived from transgenic lettuce and really completed the first in depth study of long-term antigen stability and stability under extreme conditions: while MV-H was both stable at room temperature for as long as 13 months, and at 50°C for a week. This freeze-dried lettuce-derived antigen still elicited potent immunoglobulin titers when administered as crude extracts in conjunction with MV-H DNA mixtures [57]. Recently in 2010, Pniewski et al.

took the first steps in formulating prototype orally administrable plant-derived vaccines against hepatitis B virus (HBV) surface antigen (S-HBsAg) [58]. Not only did this antigen form VLPs in vivo, but also it elicited high levels of anti-HBV specific immunity when freeze-dried antigen was administered as oral boosts to the subcutaneous prime, without adjuvant. After lyophilization, Pniewski et al. developed a tablet-based formulation using polyvinylpyrrolidone/methylene chloride as a binding agent and lactose as excipient filler. Though only a prototype formulation, this demonstrates the plausibility of this concept; however, this group further demonstrated the efficacy of lyophilized plant-derived vaccine antigens in immunization. In the cases of some nuclear transformed plants where levels of expression are often lower than those of transplastomic plants, lyophilization serves to increase antigen increasing the viability of such systems, as it was reported to produce a 7-fold increase in antigen content [57].

This process can be applied to a variety of plants and tissues including leafs, tomatoes, and potatotubers and has been shown to extend the shelf life of transgenic leafs for up to a year at room temperature [20, 56, 58]. The current commercial uses of lyophilization, as well as the obvious interests from other research groups, underscores the potential of this technique. Lyophilization will be investigated herein, in order to develop a capsule-type formulation to mediate oral delivery of plant-derived antigens. This is the first report of using lyophilization to facilitate the stabilization of chloroplast-derived vaccine antigens. Lyophilized antigens will be evaluated with respect to conformational integrity and bioactivity; furthermore, the immunogenicity of this lyophilized vaccine is investigated through a mouse immunization model. The use of lyophilization will be additionally implicated during the formulation of oral capsules.

OPTIMIZATION OF FREEZE DRYING

At the start of this study transgenic seeds were obtained from the Daniell lab seed bank

that were progeny of the original LS-HPAG and LS-CTBPins (L100) plant lines. These plants

were grown to maturity (Figure 1A.), and harvested at the time of peak antigen levels,

which was previously characterized, and immediately frozen in liquid nitrogen. At the time

of harvest, the relative water content of the fresh lettuce leaves was determined to be

69.5%, as depicted in Figure 1B. This information describes the relative turgidity of the

plant cells; while this measurement could vary between plants it is important to relate the

relative water content to the lyophilization parameter as it is a quick and efficient means of

assessing the water content of a sample. At this RWC, cells were determined to be ~95%

water, and ~5% dry cellular weight. Lyophilization treatment was optimized based on

relative gravimetric analysis of transgenic plants expressing PA and CTBPins. Incubation

Figure 1(A-C). (A) A mature, transplastomic lettuce plant (Lactuca sativa cv. Simpson Elite) expressing Protective Antigen (PA) (B) Relative water content determined in FW leaf (C) The LabConCo 4.5L Benchtop Lyophilizer used in this study.
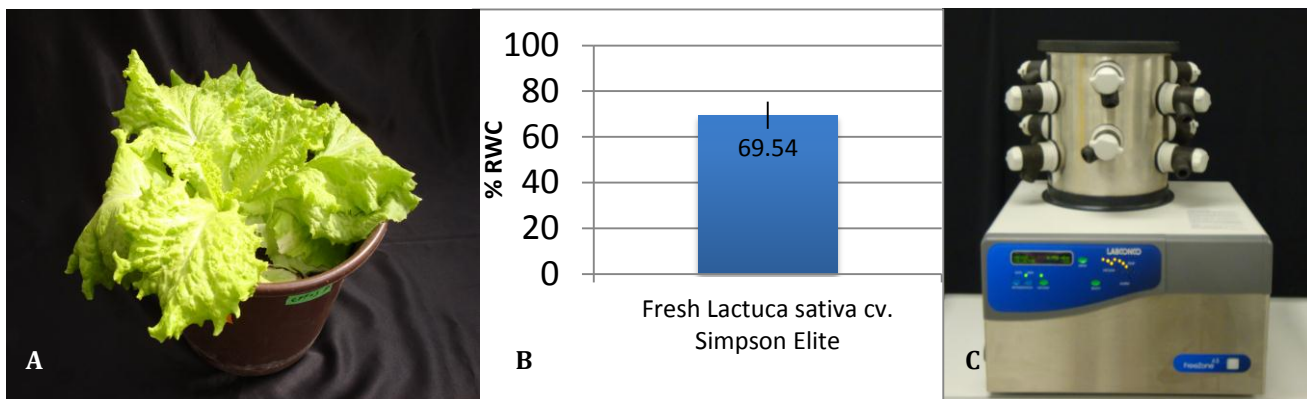
under lyophilization parameters of complete vacuum at -59° for varying durations of 24, 48

and 72 hours provided the extent of water removal with respect to time (Figure 1D). These

time points were chosen because the LabConCo manual suggested a 60-hour duration,

whereas other sources cited briefer treatments [55]. The freeze-drying system used in this

study was the LabConCo Freezone 4.5l

Benchtop Lyophilizer (Figure 1C.)

Lyophilized plant material certainly

appeared a pale color in contrast to the fresh

leaf after lyophilization (Figure 1E.) Yet the

overall integrity of the leaf was preserved.

Relative weight reduction of the leaf samples with

respect to time is given in figure 1D. While

differences in weight between the treated samples

could not be considered significant due to inherent

sample variability, it was clear that the control,

which was subjected to an oven-

drying parameter of 4 hours until constant weight
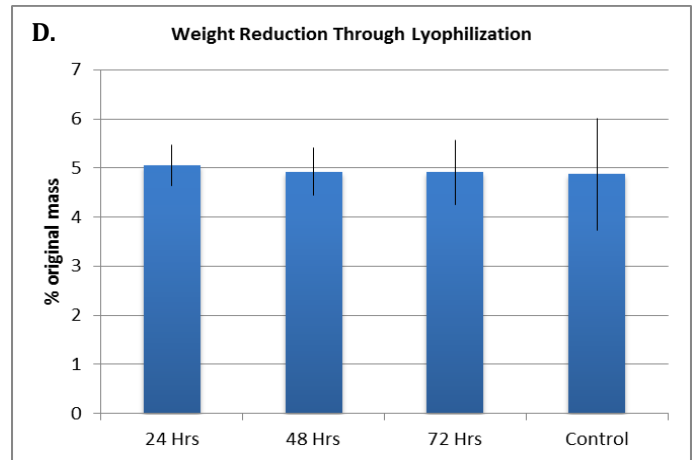
was achieved, contained no remaining

Figure 1 (D-E). (D) The reduction in sample weight provided by differing lyophilization treatments. (E) Qualitative comparison of fresh transgenic lettuce leaf with lyophilized counterpart

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water and possessed less mass than the lyophilized samples. The percentage of the total

water remaining at each time point was then extrapolated, and compared to the oven-dried

control of 0%. Plotting this data provided a trend that depicts the extent of water removal

at each time point. After 24 hours

of lyophilization, .19% of water

remained in the tissue. After 48

hours and 72 hours however,

that value was reduced to .058%

and .046% respectively. This

indicated that at least 48 hours

was required to achieve the

maximal extent of water removal.

48 hours was determined to be

sufficient to completely

lyophilize these transgenic

lettuce lines, the percent of total

cellular mass that constitutes

transgenic protein was calculated

contrasted to that of fresh

transgenic lettuce. Based on

measurements made using LS-HPAG.

F.

% Water Remaining

0.189

0.058 0.046

0.000

24 Hrs   48 Hrs   72 Hrs   Control

Lyophilization Treatmnet

G.   **% Water Removal**

99.94 99.95 100.00

99.81

24 Hrs   48 Hrs   72 Hrs   Control

Figure 1 (F-G). (F) Depicts the extent of water removal by lyophilization at each evaluated time point. (G) is the converse plot of (F), indicating the levels of water remaining in the tissues after drying.

The fresh composition of lettuce was determined to consist of 95% water, 5% cellular dry

weight, and .02% antigen. Freeze-dried LS-HPAG was conversely only .06% water, 99.62%

cellular dry weight, and therefore .33% pa. This figure gives a visual representation of the

increase in relative antigen content produced through lyophilization which will be

discussed further in later sections.

Figure 1 (H-I). This information obtained from gravimetric analysis of lyophilized tissues was used to derive (H) and (I) which provide the composition of fresh and freeze-dried lettuce materials

ANTIGEN LEVEL STANDARDIZATION

In order to work with these proteins in a quantitative manner, standarization of antigen levels was necessary. For fresh weight (FW) and freeze-dried (FD) LS-HPAG, the Enzyme-Linked Immunos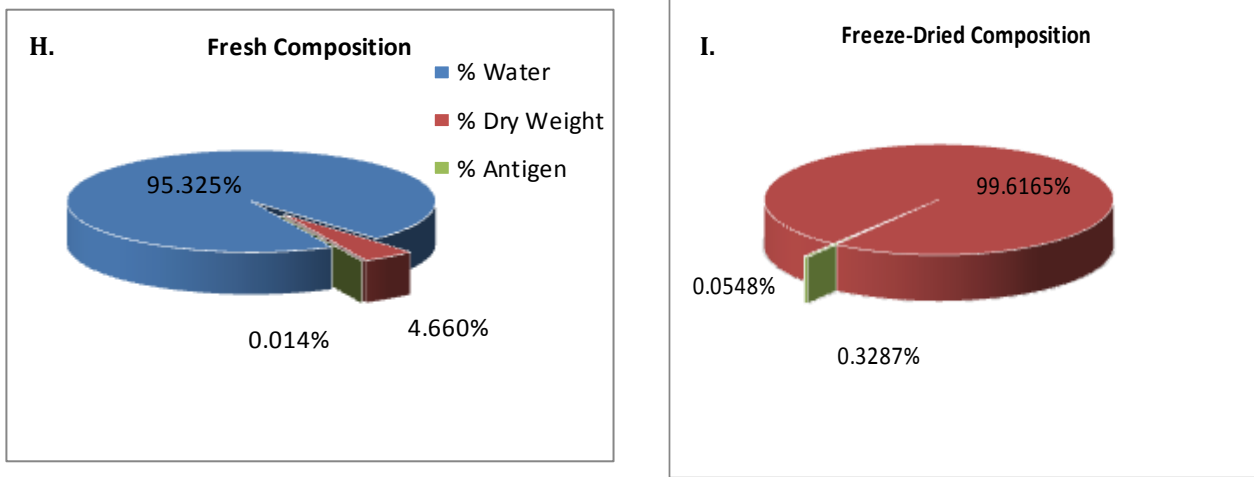orbent Assay was used to quantify PA levels by correlation to a purified standard of *B. anthracis* derived PA. The Bradford assay was used to determine levels of total protein. FW LS-HPAG expression was measured at 3.5-4.5% TSP, which equates to approximately 140 micrograms per gram of leaf. The DW composition was nearly 24-fold higher, indicative of the considerable reduction in relative water content. Due to the stable state of the lyophilized lettuce, further homogenization was possible using a simple coffee mill; this device was capable of producing coarsely crumbled leaf pieces (Figure) or refined leaf powder when grinding time was extended and leaf was passed through a sieve.

A. Protein Content of Transgenic Lettuce

Legend: Protective Antigen, Total Protein

B.
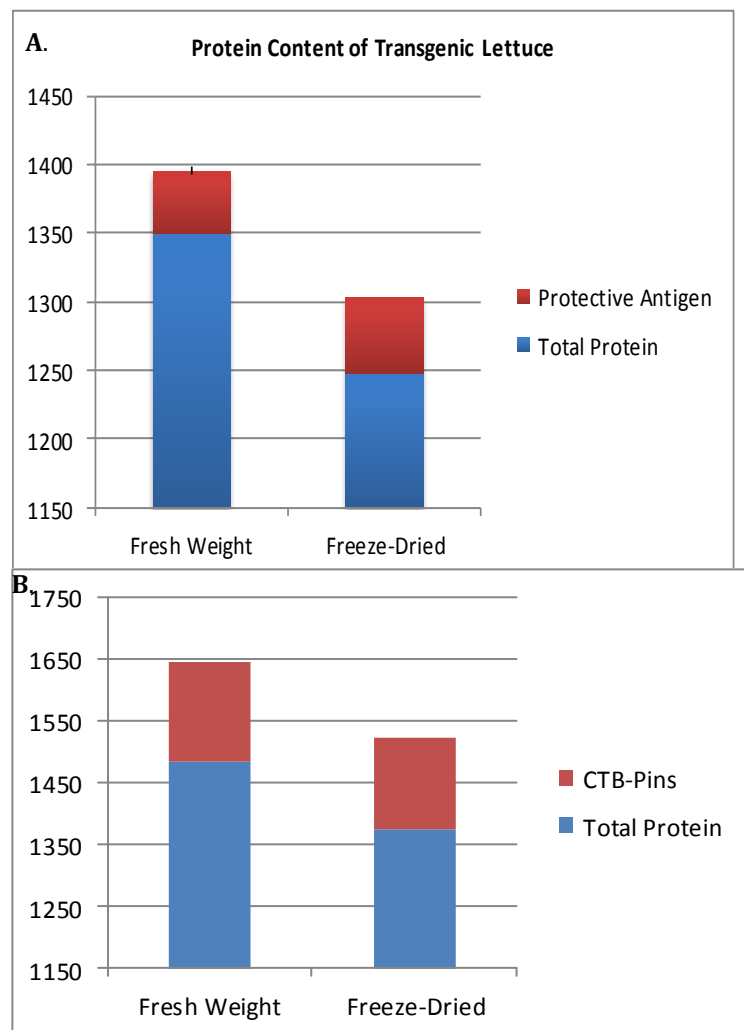
Legend: CTB-Pins, Total Protein

Figure 2 (A-B). (A) Depicts the protein content of FW and FD LS-HPAG tissues; a TP expression of 3.5-4.5% TSP was observed in this plant line (B) Protein expression profile of LS-CTBPins; expression in this plant line was observed at 10-11%.

This highly homogenous sample set enabled very accurate estimation of the entire batches'

antigen content as is demonstrated through the normalization of LS-HPAG antigen content

(Figure 7). The normal distribution of measured antigen content in lyophilized samples

becomes more precise, and the standard deviation decreases. Quantification of CTB-Pins

through ELISA is not possible due to the insoluble nature of this particulate antigen;

therefore, densitometric analysis in conjunction with the Bradford assay was used to

estimate levels of expression in this line. Duplicates of serially diluted CTB-Pins extract

were boiled for 15 minutes to denature tertiary structures and oligomers and was loaded

in SDS-PAGE and run alongside CTB standards. Spot density evaluation enabled for

accurate estimation of CTB-Pins Levels, which provided levels of expression around 10%
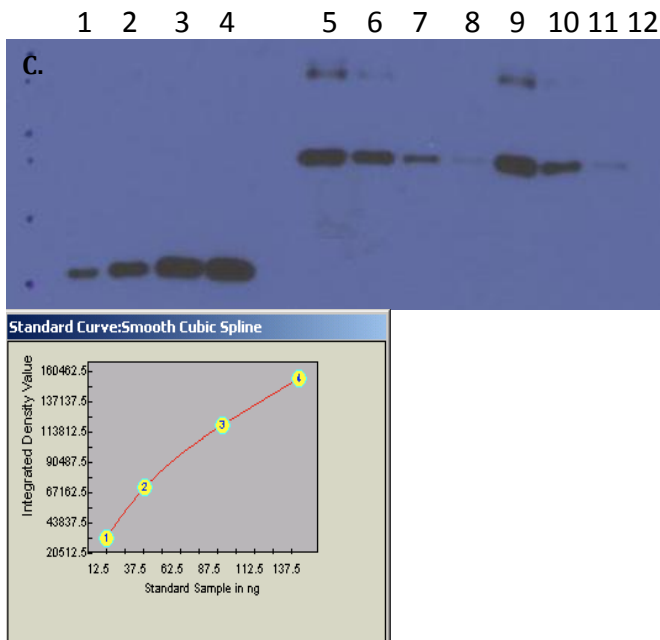
TSP, or an equivalent of 13.3 mg/g.

Figure 2(C) (C) Western blot of CTB standard and CTBPins extracted (serially diluted) used in the quantification of this plant line; the standard curve obtained through densitometric analysis is provided.

LONG TERM ANTIGEN STABLIZATION

The shelf life of plant-derived antigens is of major concern in relation to the distribution of biopharmaceuticals to rural areas. Inures where shipments may be infrequent, and cold-chain storage is simply unavailable, the shelf life of plant-derived

Freshly Lyophilized **A.**   1 Year Old

antigen is highly important and could affect the proportion of the population which receives proper treatment. After lyophilization, plant-derived antigen was evaluated for long-term stability through SDS-PAGE using rabbit anti-PA antibody. After one year of room temperature storage in the dark, the appearance of the freeze-dried material had decreased levels of green pigment. It appeared paler and yellow than freshly lyophilized tissues (Figure 3A). However evaluation of antigen integrity demonstrated good preservation of both PA and CTB-Pins. 3 ug loads of FD LS-HPAG of two, four, and six months of age were compared to FW LS-HPAG, and there were no observable degradation products in the lyophilized samples. Furthermore, 6-month-old samples that were differentially treated in regards to
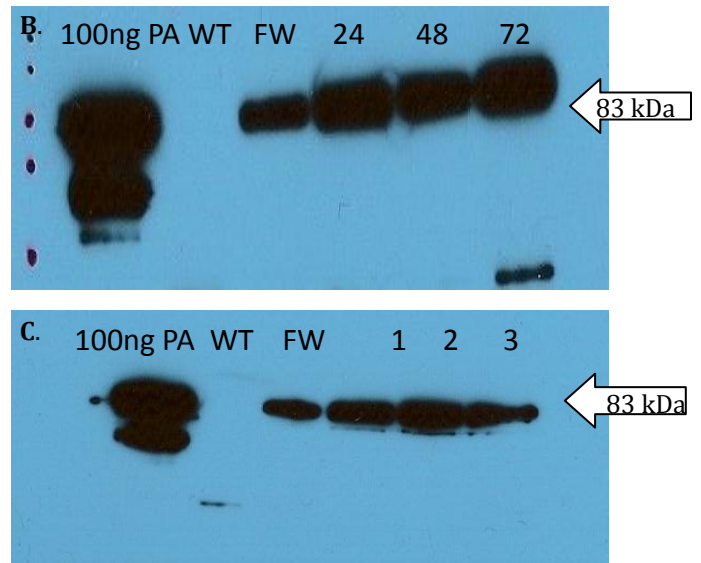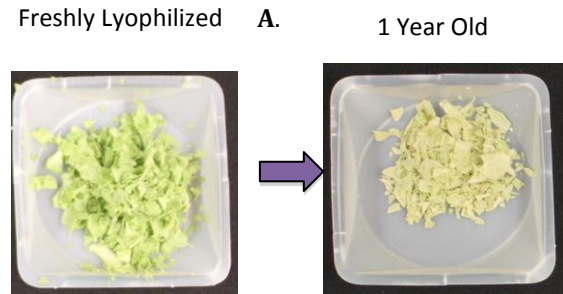

Figure 3 (A-C). (A) demonstrates the qualitative change in appearance of lyophilized leaf material over the course of 1-year. (B) 6-month old tissues lyophilized for varying durations evaluated for antigen stabilization. (C) FD LS-HPAG that is 2, 4 and 6 months old respectively (1,2,3).

lyophilization duration (24, 48, and 72 hour) were evaluated through SDS-PAGE. Again, no observable significant difference exists between the FD and FW samples. Interestingly however, 24 Hrs provided levels of stabilization indistinguishable from those of 48 and 72-hour treatments, suggesting that maybe this treatment was indeed sufficient. Evaluation of one-year-old FD LS-CTBPins provided similar results, demonstrating the efficacy of this freeze-drying process in maintaining antigen integrity.

FW and FD PA were subsequently evaluated through comparative SDS-PAGE and Native (non-denaturing, non-reducing) gels to examine potential conformational changes, or sugar linkages that occurred during lyophilization. Both FW and FD PA migrated similarly on gels regardless of their denaturing or native properties. This data suggests that no major conformational modifications are bestowed on PA during the freeze-drying process, which further authenticates this approach's benefits for plant-based vaccines.
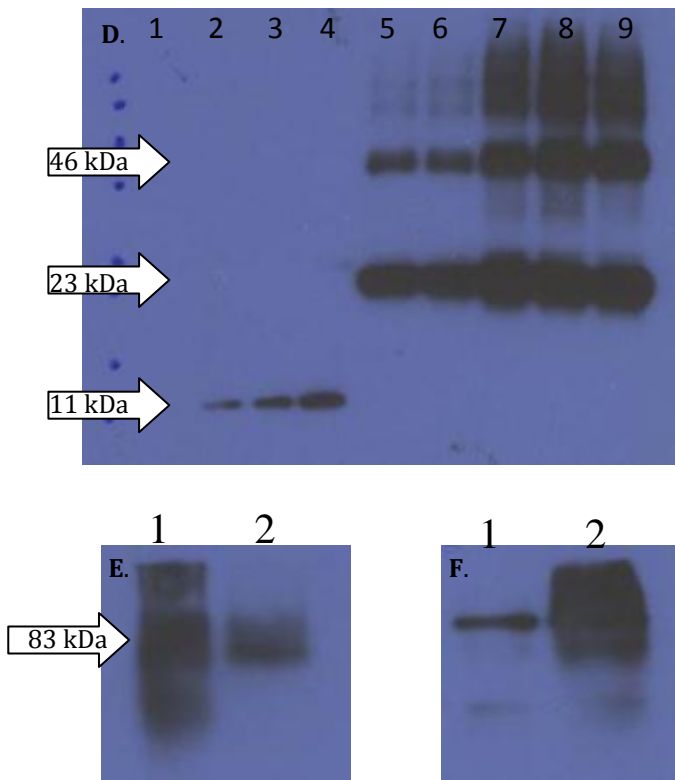

Figure 3 (D-F). (D) Displays the stability of 1-year old FD LS-CTBPins (7-9), in comparison with FW LS-CTBPins (5-6) and CTB standard (1-4). (E) Demonstrates that FW and FD PA migrate similarly during SDS-PAGE. (F) Demonstrates that FW and FD PA migrate similarly during native gel electrophoresis, indicating that now protein modification occurred during lyophilization.

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PHOTOSENSITIVITY OF LYOPHILIZED MATTER

The cell-death induced by freezing immediately jeopardizes the integrity of chlorophyll within the light harvesting complexes on the plant cell membranes. Therefore, the gradual depletion of green vitality has previously observed in Figure 1E is to be expected. However, the observation that a FD sample left on the desk was being bleached by the sun led to the investigation of potential antigen sensitivity through the same oxidative mechanism. A sample of FD LS-HPAG was exposed to direct sunlight for 3 months. First, levels of chlorophyll A and chlorophyll B were evaluated through methanol/acetone extraction and evaluation of absorbtion at 650/640. It was observed that not only had the freeze-drying process significantly depleted endogenous chlorophyll, but the photobleached sample had been completely abrogated. To
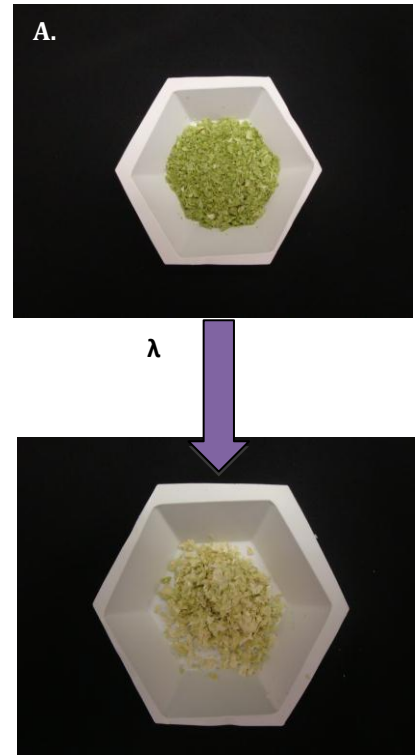
Figure 4 (A,B). (A) A major qualitative change in lyophilized material was observed in response to prolonged sun exposure. (B) Chlorophyll is partially degraded during lyophilization, but remaining stores in lyophilized tissues are sensitive to light-exposure.
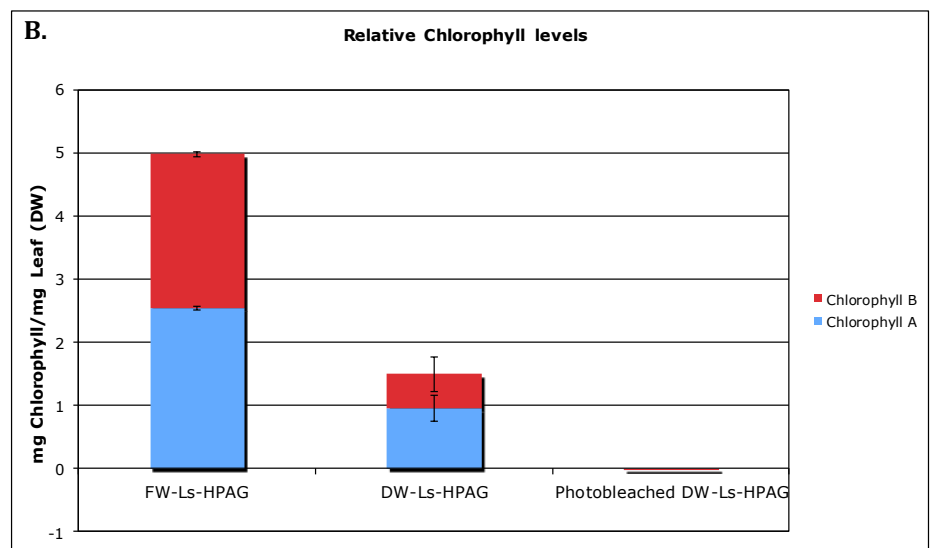
investigate the potential photo-oxidation of Protective Antigen, extracts of the

photobleached sample were compared with those of FD and FW tissues in order to contrast

the antigen integrity. While SDS-PAGE analysis suggested slight degradation of target

protein, ELISA refuted any significant difference between the PA content of these tissues.

Together, this data means that while endogenous secondary metabolites of the plant may

be sensitive to photooxidative mechanisms, the target antigen PA was uneffected by light,

and was therefore stable under minimalistic storage conditions.

Figure 4 (C,D). (C) depicts stability of PA in photobleached samples in comparison to FW (1-3) and 48-Hr FD (4-6) controls. (D) quantification of PA in the same tissues through ELISA provided no significant difference between FD LS-HPAG and the photobleached sample.

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PROTECTION OF ANTIGEN FROM GASTRIC DIGESTION

Of the major impediments to oral immunization is the digestibility of target proteins in the human gastrointestinal tract; the viability of this vaccine technology depends on successful delivery of target protein to the small intestine. Plant cells, impervious to human d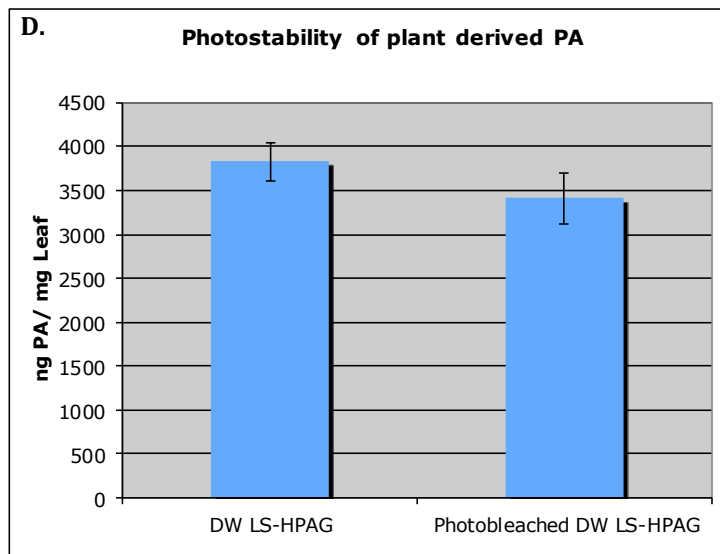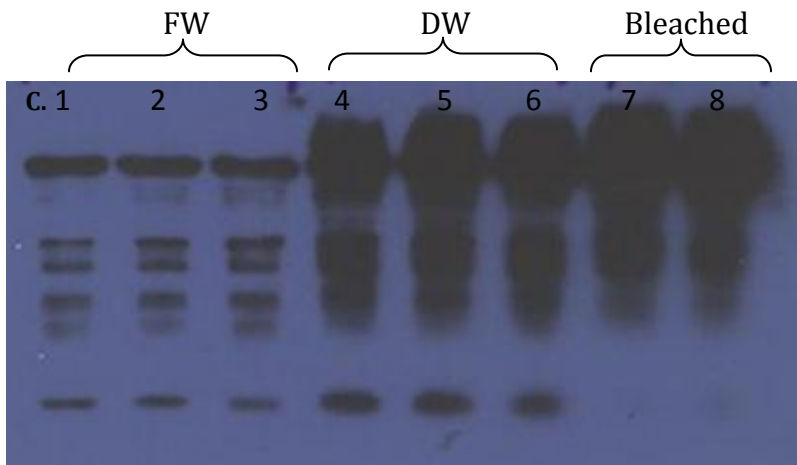igestive enzymes, accordingly protect protein from proteolysis through a mechanism termed bioencapsulation. Bioencapsulation, though long argued for by plant-based vaccine proponents, has not been previously demonstrated in plants.

Recent efforts to characterize certain defining characteristics of food allergens and to evaluate allergenicity of transgenic food proteins has resulted in the development of an accurate *in vitro* representation of gastric digestion, as a means to evaluate a protein's potential to persist through the stomach and subsequently induce an IgE mediated allergic reaction in the intestinal mucosa. The survival of the protein through Simulated Gastric Fluid (SGF; 0.2% NaCl, 0.7%HCl, 0.32%Pepsin, pH1.2, 37°C) indicates a proteins potential role as a food allergen. The SGF assay may therefore be applied to plants bearing antigen subunit vaccines to asses the protection conferred by the plant cell vector through bioencapsulation.

Plant-derived protein was be exposed to digestive conditions in a 37°C water bath for a one-hour time frame with samples taken at time points (0, 5, 30, 60, 300, 900, and 3600 seconds). At each representative time-point, sample digestion was quenched through the addition of Tris-Cl buffer supplemented by a protease inhibitor cocktail (Roche) prior to protein isolation and analysis via SDS-PAGE and Western Blotting. This reaction quench raises the pH such that Pepsin assumes an inactive conformation, and the protease inhibitor further inhibits target protein degradation. While Pepsin possesses no catalytic

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ability to degrade cellulose, the combined effects of acid hydrolysis, hypertonic pressure,
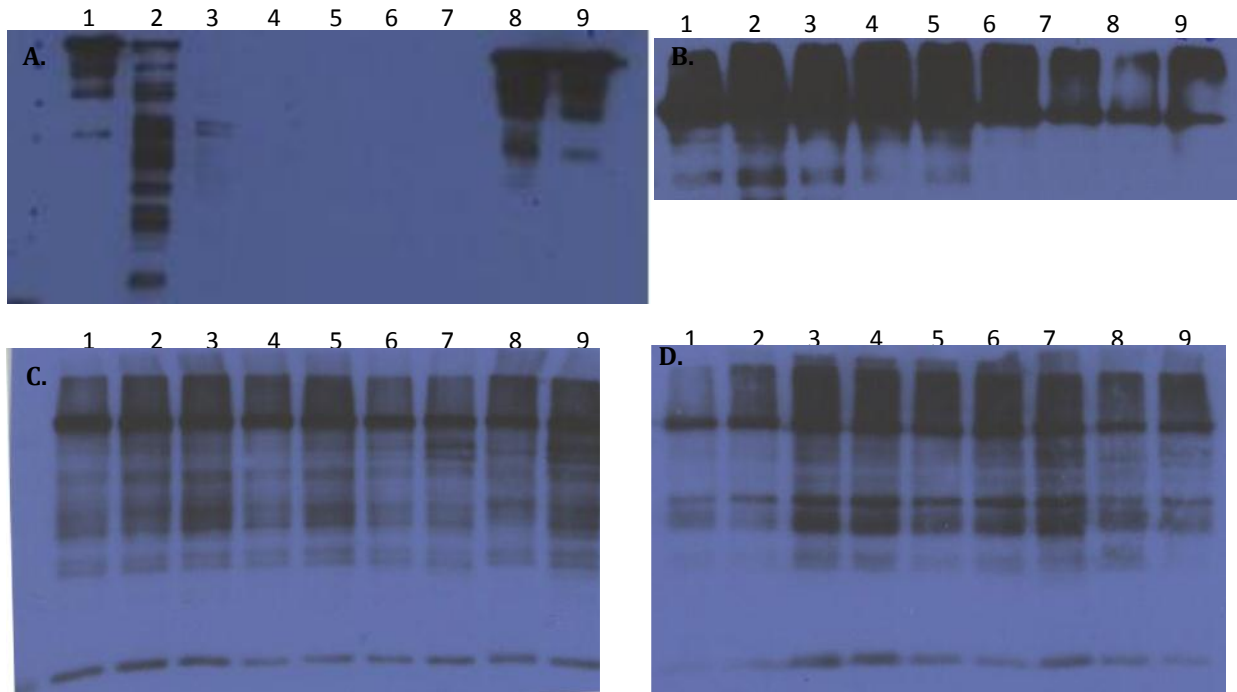
Figure 5 (A-E). (A) SGF assay on purified, bacterial PA; while severe degradation is observed in the first 30 seconds no antigen is detectable beyond 1 minute. (B) Tobacco-derived PA, in the SGF assay; tobacco provided substantial antigenic protection through bioencpasulation. FW (C) and FD (D) LS-HPAG also demonstrated significant levels of antigen protection like tobacco. Loads: 1-(-) control, 2- 5 sec, 3- 30 sec, 4- 60 sec, 5- 300 sec, 6- 900 sec, 7- 3600 sec, 8- (-)F, 9-Q.

and activation of endogenous protease machinery could lead to degradation of target

protein.

Despite these digestive pressures, plant-derived bioencapsulated PA was protected

throughout the one-hour digestion time course, while purified PA was rapidly degraded

within one-minute. FD material did not experience degradation of target protein indicating

that the lyophilization process maintained membrane integrity such that too it was capable

of facilitate intestinal delivery of antigens, despite the freezing-steps implicated in the

treatment process. Furthermore, the protection conferred by lettuce cells was qualitatively

similar to that provided by tobacco cells in the. This observation authenticates the potential role of lettuce as an edible vector for oral immunization. The representative image provided in Figure 5E displays the observed color change promoted during digestion. The depletion of the green color in this image is representative of the gradual destruction of cell walls, and consequently the light harvesting complexes, which results in the gradual depletion of chlorophyll. The differential preservation of the green color, indicative of chlorophyll, was rapidly depleted in time point samples, however the quenched sample (Q) which was supplemented with pepsin neutralization before exposure to the digestive juices maintained green color despite 1-hour incubation at 37℃. While (-) and (-)$_F$ (treated with pepsin⁻ SGF) demonstrated the same color change in a time dependent manner, this data indicates that the synergism of acid hydrolysis and osmotic potential within the stomach is sufficient to drive the disruption of cell walls.
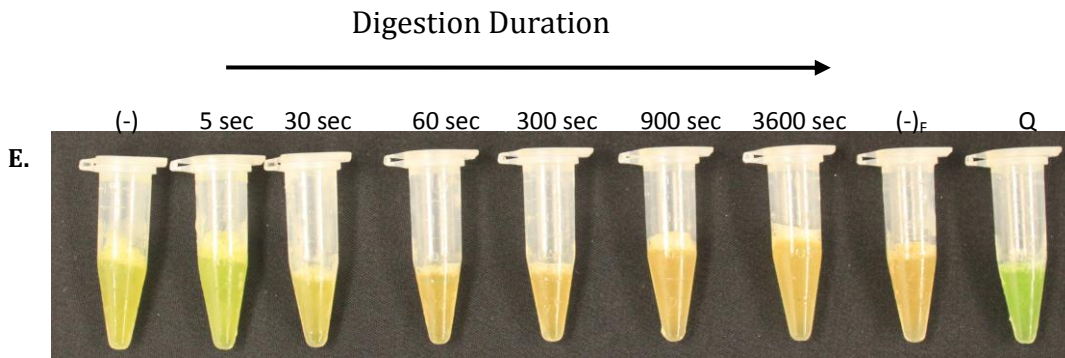
Figure 5 (E) Qualitative representation of the effects of digestion of plant tissues bearing antigen. (-) samples represent incubations in acidic SGF in the absence of pepsin, Q sample represents SGF/Pepsin mixture that was neutralized prior to 1 hour incubation. Time points from 5 seconds to 1 hour.

IMMUNOGENICITY OF LYOPHILIZED PLANT DERIVED PROTECTIVE ANTIGEN

While lyophilized PA had maintained its structural identity through lyophilization, and previous publications demonstrated the bioactivity of plant derived PA, a mouse immunization model was adopted to evaluate the immunogenicity of lettuce derived PA, as well as that of FD-PA. Using an immunization regime consisting of a subcutaneous primovaccine followed by oral boosting with plant derived PA, mice were immunized against anthrax. Induction of IgG in serum was monitored over the course of the immunization experiment. FD LS-HPAG immunized mice produced higher antibody titers than its FW counterpart; additionally, sera from mice immunized with FD LS-HPAG demonstrated the ability to neutralize LT in the toxin neutralization assay. As expected, mice immunized by FD PA demonstrated the highest survival rate (40%) when challenged by $1.5LD_{100}$ cfu aerosolized spores, in comparison to FW immunized mice (20%), and FW immunized mice who didn't receive the primovaccine (0%). The group that never received
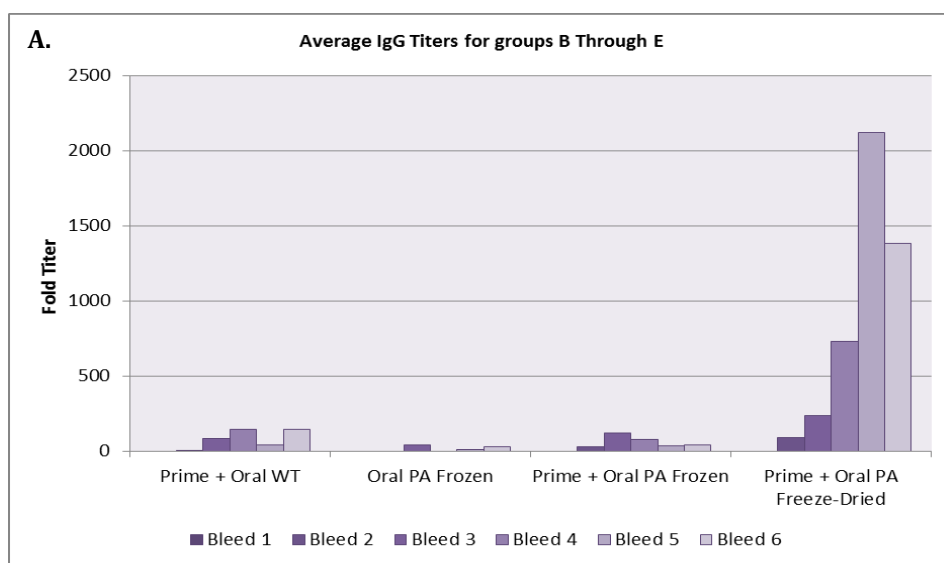
Figure 6 (A). IgG titers of test groups in mouse immunization model after 12-weekly gavage boosts; plant-derived antigen that was preceded by a subcutaneous prime initiated the most potent immune response.

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the subcutaneous prime likely promoted the induction of a TH2 cell mediated Treg

response upon oral antigen uptake. This data indicates that immunogenicity is at least

preserved through lyophilization; whether lyophilization helps increase the

immunogenicity of antigens remains to be seen, however previous reports have suggested

this concept [58]. Lyophilization is therefore an effective means to stabilize plant-derived

vaccine antigens.

Figure 6 (B-C). (B) Evaluation of only the group that was immunized by FD LS-HPAG; maximal response was reached by week 10. (C) Toxin neutralization assay data of sera obtained from the mice of this immunization study. Data obtained agrees with previously characterized IgG titers.
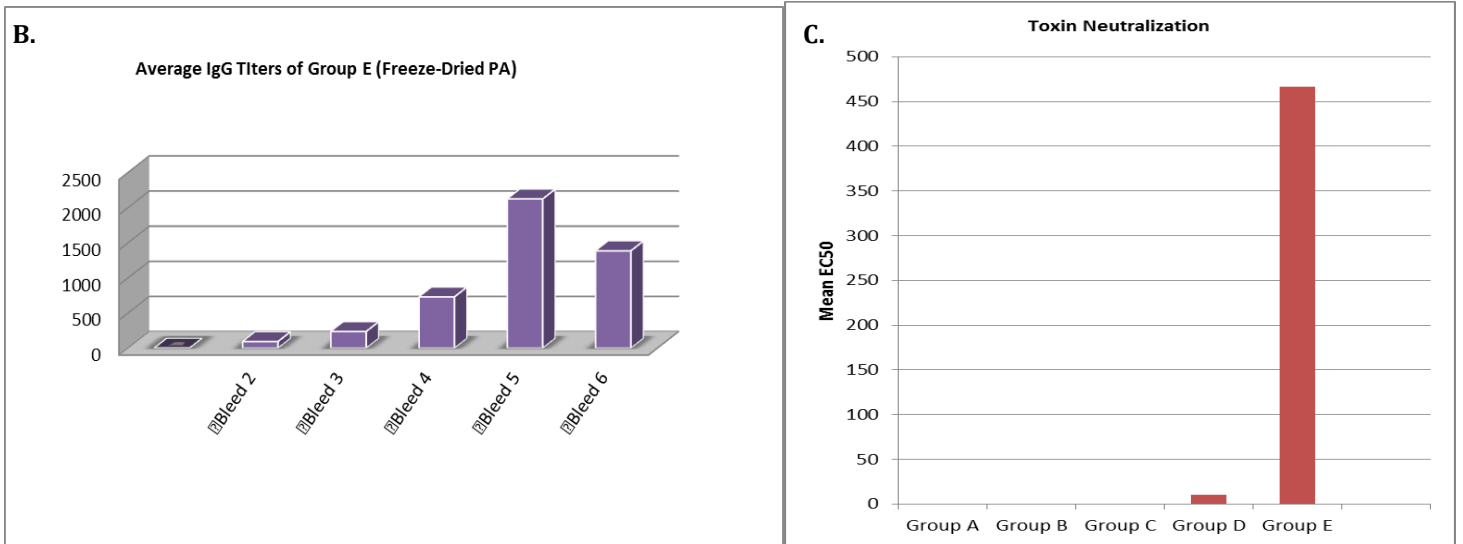
D.
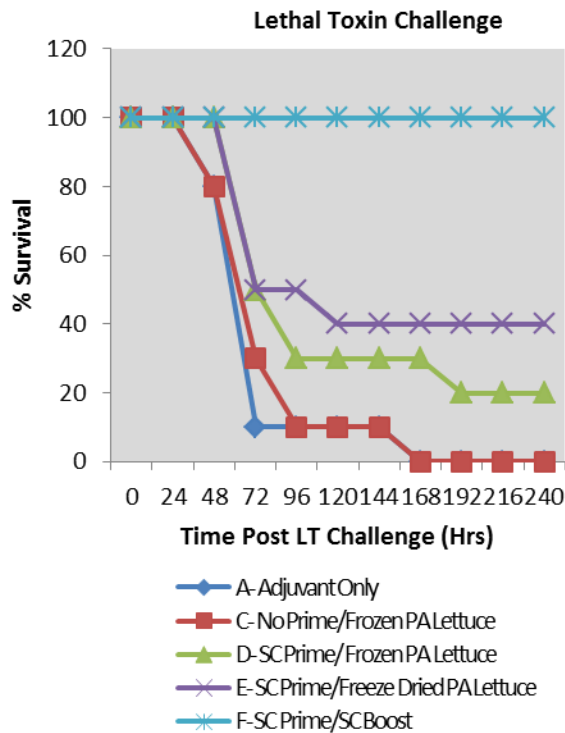
Figure 6 (D). Lethal toxin challenge of mice immunized by different combinations of FW LS-HPAG (prime vs. no prime) and FD LS-HPAG. S.c. injection elicited the highest immune response and accordingly provided the most protection, while mice boosted by FD LS-HPAG were bestowed with the second most effective response geared towards anthrax.

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FORMATION OF ORAL CAPSULES

As this field develops, it is becoming more apparent the original concept of "edible vaccines" may never be achievable. It is essential that immunization procedures be characterized and strictly followed in order to properly confer immunity and to avoid the induction of antigen tolerance. Therefore, much control must be exerted over the delivery of plant-derived vaccines and material must be produced in a consistent manner. To do this, antigen-bearing material must be formulated into capsules or tablets for the efficient and regulated distribution of this type of vaccine. When considering the many options available for producing these objects, there are many possibilities of formulations, stabilizing agents, or additives that could increase the efficiency of delivery and thus the efficacy of plant-derived vaccines.

Capsules are an inexpensive and frequently used method of producing plant supplements or vitamins. In contrast to tablets which require binding agents and expensive production units, capsules require minimalistic processing of the leaf and can tolerate mildly heterogeneous leaf powders. Instead of grinding by mortar and pestle, a simple
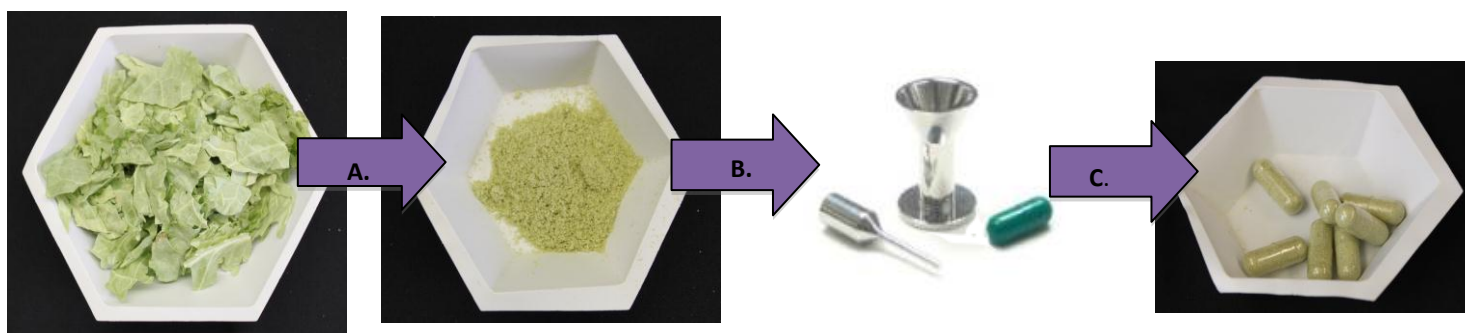
Figure 7. The formation of encapsulated plant-derived biopharmaceuticals. Processing of crude, lyophilized lettuce (A) by a coffee mill produced a finely ground powder that was subsequently refined by passage through sieve. This preparation was used to fill capsules to produce a novel means of antigen delivery to the gut mucosa

used gelatin capsules that will simply release in the stomach, complex options are available with targeted releases; one example is a pH dependent release system wherein the ingested capsule does not dissolve until it reaches the pH of the intestinal lumen. Additionally, other biopolymers such as chitosan have demonstrated potential to protect protein-based therapeutics during oral delivery. Capsules made from such polymers could improve the bioencapsulative protection of target protein. Simple powdered adjuvants such as crude saponins may be processed into these capsules to promote the formation of an oral immune response. Lactose and other inert excipients may be added to produce specifically catered antigen doses, in order to specialize delivery for either children or adults. Lyophilization enables easy and stable processing of plant-derived vaccine antigens into formulations conducive for oral delivery. Further improvements of this proto-type oral formulation may be studied, using lyophilization of plant-material as the means to generate stable recombinant antigens.

CONCLUSIONS

The inherently high water content of lettuce cells that constitutes ~95% of its fresh weight generates an environment unfavorable to the accumulation of target protein in transgenic plant systems. Lyophilization was therefore applied to transgenic plant tissue with the intent to preserve target protein in a stable form through the removal of water. Characterization of the relative water content of harvested lettuce provided that cells of *Lactuca sativa* leaves maintain turgour pressure at only 70% of their maximal capacity; however, this large ratio of water to cellular dry weight dilutes target proteins and provides conditions favorable for proteolysis. The application of lyophlization is known to efficiently reduce the water content of plant material and was investigated for its potential to abrogate the drawbacks of plant-tissue vectors.

While sources in literature suggested that lettuce was particularly recalcitrant in lyophillization, and that a 60-hour treatment might be required for optimal drying, 48-hours of lyophilization was shown to be sufficient in the experiments herein. Oven-dried control samples suggested that the cellular dry-weight of fresh plant tissues was roughly 4.6% of the overall fresh weight. Gravimetric analyses of lyophilized samples before and after treatment provided the reduction in weight generated by each time point. While simply examining the reduction in weight, the differences between different time points provided no significant differences; however, a cross-comparison with controls allowed the determination of the percent of initial water remaining in lettuce at each time point, and conversely the percentage of water removal accomplished by that point. While data indicated that 24-hours reduced water levels to .2% of the original level, and therefore was

99.8% efficient, 48- and 72-hours provided a further 4-fold decrease in water content. These periods of treatment were found to reduce the weight of tissue samples to 4.92% and 4.91% of their original weights respectively. This corresponded to .058% and .046% of total remaining water suggesting that 48-hour treatment was 99.94% efficient, and 72-hours was 99.95% efficient. While the differences between the two and three day treatments were minimal, they provided a clear reduction in weight over those samples treated for only one-day. The 48-hour treatment was therefore chosen as the optimal treatment for the transgenic lettuce lines used in this study. As described previously, lettuce tissues lyophilized for 48-hours still contain .058% of their original water content; this drastic reduction in water content increased the cellular dry-weight of fresh material from 4.6% to 99.6%. This increase additionally corresponded to a 20-fold increase in the target protein content in plants bearing vaccine antigens.

In fresh-weight tissues, high-water content produces variability of antigen levels with respect to biomass and occludes easy normalization of antigen levels. Removal of water through lyophilization facilitated easy homogenization of freeze-dried tissues. While homogenization of fresh tissue generates cell lysates with rapid proteolysis, the homogenization of freeze-dried tissues in a coffee mill produced a finely ground powder characterized by stable antigen. This homogenization step can facilitate normalization of antigen batches, as was demonstrated herein. Despite the 20-fold increase in antigen content, variability across samples was reduced when transgenic protein levels were determined through ELISA or densitometric analysis.

An evaluation of the integrity of lyophilized plant-derived antigens after extended periods of storage at room temperature demonstrated their stabilization through

lyophilization. 48-hours of freeze-drying generated stable antigens for over a year, under desiccated storage parameters. Comparison of protein extracts obtained from one-year old lyophilized protective antigen through SDS-PAGE and non-denaturing, non-reducing native poly-acrylamide electrophoresis showed no significant difference between fresh and lyophilized antigen. This verifies that antigen conformation is maintained during the freeze-drying treatment, which suggests that immunogenicity of the vaccine antigen is maintained. This further demonstrates that no linkages between the target protein and reducing sugars are induced via the removal of water, as some sources have suggested as a possible drawback of lyophilizing proteins within plants.

Though lyophilized plant-derived antigens were stable under specific storage parameters, samples exposed to sunlight demonstrated the photosensitivity of the crude product. A depletion of green pigment was observed, which was subsequently correlated with a depletion of endogenous chlorophyll. A comparison of chlorophyll levels between fresh lettuce, lyophilized lettuce and the photobleached samples characterized the effects of lyophilization of other endogenous metabolites. Lyophilization induced a dramatic decrease in chlorophyll levels, likely due to the rapid freezing by liquid nitrogen associated with the freeze-drying process. Freezing ultimately drove the rupturing of cellular membranes, which resulted in the disruption of light harvesting complexes (LHCs) within plant cells. While unstable outside of the LHCs chlorophyll rapidly oxidizes, the likely occurrence that provides the observed decrease in endogenous chlorophyll. Chlorophyll in photobleached samples was below detectable levels, which appropriately describes the observed qualitative change in the plant material. This observation led to investigation of transgenic antigen stability with respect to photooxidation. Lyophilized PA demonstrated

no such sensitivity and was found to be stable in lyophilized tissues even after three months of direct sunlight exposure.

While the survival of gastric digestion is critical to the success of the oral delivery of any therapeutic protein, the highly proteolytic environment of the stomach occludes efficient oral delivery of proteinaceous therapeutics. Bioencapsulation by plant cell walls has been openly discussed as a viable mechanism for antigen delivery to the gut, yet experimental demonstration of this phenomena has yet to occur. The simulated gastric fluid assay was applied to evaluate the potential of plant-cell vectors to deliver antigen in this context. Purified antigens were used for assay optimization and standardization; non-bioencapsulated antigen was rapidly digestion within one-minute of exposure. Conversely, plant-derived antigens were shown to be stable and detectable via immunoblotting after one-hour of exposure to digestive conditions. There were no significant differences conferred by lettuce versus tobacco cell walls, demonstrating that lettuce is a viable alternative to this platform, despite the fragility of this plant. Digestion produced a fade in visible chlorophyll that directly correlated to the duration of digestion, which suggests LHC and by association the degradation of cell walls. This exhibits a protective property conferred solely by the chloroplasts as no deterioration in antigen is observed and indicates that further investigation into chloroplast stability during digestion should be investigated. The SGF assay utilized herein accurately assesses the potential for oral protein-delivery through this system and substantiates the proof of concept for the oral delivery of plant-derived biopharmaceuticals.

The immunogenicity of freeze-dried plant-derived antigens was maintained for up to a year, corresponding to the preserved integrity of the protein. Mice immunized by oral

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boosting of lyophilized lettuce derived PA after a subcutaneous primovaccination developed a measurable 2000-fold titer induction of IgG molecules in response to PA. This significant response increased with each subsequent oral delivery of plant-derived PA, demonstrating that the oral delivery of antigens bioencapsulated by plant cell walls is an efficient means of boosting immunizations. However, the lack of immunogenic response in mice that received oral boosting without primovaccination underscores one of the main impediments to purely oral immunization strategies. The general induction of tolerance against antigens within the gastrointestinal tract occludes efficient immunization without prior priming of an adaptive immune response. The addition of adjuvants that promote the induction of immunity could improve this efficiency; however, the mechanisms that regulate mucosal immunity are poorly understood. Therefore, rational design of therapeutics is difficult. Furthermore, the sharp contrast of survival rates in animals which received subcutaneous immunization (100%), against animals that were boosted orally by freeze-dried PA (40%), implies that the uptake of antigen within the intestinal mucosa is inefficient. In previous studies that utilized receptor-mediated forms of delivery, such as the CTB vaccine against cholera, the titers of orally immunized animals were more consistent with that of sc. injected mice. The titers induced via receptor-mediated delivery methods were more consistent across animals, whereas mice that received orally boosted PA were very disparate between mice. This demonstrates the requirements for improved antigen delivery methods to the gut; the development of other receptor-mediated approaches that are compatible with large proteins such as PA could drastically improve animal survival rates. Another research group with a probiotic, *Lactobacillus acidophilus*, approach to PA immunization tackled this issue by incorporating a dendritic cell targeting

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peptide, which they identified through reverse-phage display libraries, as a fusion antigen to mediate antigen delivery. Strikingly, PA when delivered alone induced only 40% survival rates in animal, in agreement with our findings. However, the incorporation of the DC-targeting domain improved survival by two-fold, conferring protection to 80% of animals. This study highlights the importance of improved methods in mucosal antigen delivery. A final observation with respect to the mouse-immunization is that the freeze-dried PA induced higher titers than its fresh counterpart. Lyophilization could have increased the immunogenicity of the antigen by changing its conformation in some way, such as forming particulate antigen. However, it is also possible that lyophilization only altered the digestibility of this antigen. In either case, the differences in immunization efficiency by fresh and freeze-dried plant-derived antigens should be investigated further,

The dried state of the plant material after lyophilization facilitated refined homogenization of the plant material, while maintaining its stability at room temperature. In addition to normalizing the batch antigen content of this material, the refined form of the plant material could be quickly processed into gelatin-based capsules, as the vehicle for oral delivery. Such capsules would allow for the inclusion of excipient additives. Fillers such as lactose may be used to develop specific antigen doses and specialized vaccines for adults or children. Hygroscopic additives facilitate the swelling of the capsules and the release of the plant contents, and use of pH-specific capsules would enable targeted release directly to the small intestine. All such improvements to this technology may now be examined due to the formulation of this plant-derived antigen after lyophilization.

Lyophilization proved to be a useful means of preserving plant-derived vaccine antigens. In addition to being stable during prolonged room-temperature storage,

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lyophilization facilitated the processing of crude harvested-leaves into a refined capsule, while simultaneously eliminating bacterial flora and providing a 20-fold increase in antigen content. These effects synergize to eliminate a significant portion of downstream production costs; therefore, this technique could lower the general cost of therapeutics or vaccines to make the more accessible to the general public.

METHODS

Lyophilization optimization.

Frozen tissue was subjected to partial homogenization, mainly to disrupt large pieces of leaf in order to ensure relatively equal surface-area to volume ratios. When this was not done drying process produced dark patches of concentrated pigments localized at the interior of the leaf, likely a result of gradual depletion of water from the peripheral edge of the leaf (Not shown). This crumbled material was then transferred into either 50-mL or 15-mL falcon tubes and sealed with 3M Millipore Medical Tape. This tape possesses small pores designed provide air to healing wounds, but was used in this situation as a barrier transmissible by evaporating water yet not by plant material. Samples were transported to the lyophilizer on liquid nitrogen and treated for varying durations of 24, 48 and 72 hours. Optimization based on %water removal, measured through relative gravimetric analysis. Lyophilization occurred at -58 C, under vacuum conditions. Optimization was completed through comparative gravimetric analysis.

Protein extraction

Protein extraction was completed at a ratio of 100mg FW leaf/ 300 ul of Plant Extraction

Buffer, or at a ratio of 5mg DW leaf/300 ul PEB. PEB components include NaCl, EDTA, 1M

Tris HCl pH 8.0, tween-20, β-mercaptoethanol, dithiothreitol, Sodium dodecyl sulfate,

phenylmethylsulfonylfluoride and protease inhibitor cocktail. Tissue homogenization was

completed through sonication at 5 W for 3 bursts of 5 seconds each.

Western blotting:

SDS-PAGE gels were cast in varying mixtures of Tris-Cl and acrylamide. Protein samples were mixed with either 2X Laemelli or 6X Laemelli reducing buffer, before being boiled for 10-15 minutes. Samples were immediately loaded onto gel and run. After transfer to nitrocellulose membrane, western blotting protocol was completed. Briefly using a 3% PTM solution the membrane was blocked for 1 hour before the addition of primary antibody for an overnight incubation (1:3000, PA: rabbit anti-PA, CTBPins: rabbit anti-CTB). Secondary antibody (1:5000 goat anti-rabbit, Southern Biotechnology) was added for 1.5 hours have rinsing off unbound primary antibody. Lastly, chemmiluminescent (Thermo Scientific) substrates provided for film-based detection of protein signals.

ELISA

Quantitative: Protein extract homogenates are centrifuged to remove particulate matter, and the resulting supernatants are diluted serially around levels of 1:5,000. These dilutions are related to a standard curve of purified PA in order to derived an accurate estimation of the unknown amount. This comparison occurs through a similar immunoblotting approach as the technique described above, which utilizes a primary antibody to recognize antigen, followed by a secondary antibody conjugated to Horse Radish Peroxidase (HRP). Antigen is first coated on the plate overnight in a 100 ul volume. Then blocking, primary and secondary all occur for one hour at 37°C with intermittent washes by water and phosphate buffered saline. Signal detection here is accomplish with the substrate 3,3'-5,5' tetramethylbenzidine which gets converted to a blue compound by HRP, but then assumes a yellow producing conformation up stopping the substrate reaction with 2 normal sulfuric acid. The plate is read at 450nm to detect the yellow color.

Antibody Titers: In this catenation of the ELISA, the plate was first coated with purified antigens, and test mouse sera was evaluated for its ability to recognize PA. Mouse Fc regions were then recognized by rabbit anti-mouse secondary antibodies, and plate was read at 450nm.

Bradford Assay

In the Bradford assay protein extracts are serially dilute with known concentration of bovine serum albumin (BSA), in order to determine the concentration of total protein in a mixture. Therefore a derivative of coomassie blue is used as the detection agent here, as it interacts nonspecifically with aromatic amino acids. The level of blue intensity between the defined standard and the unknown may be correlated by reading at 595 nm.

Densitometric Analysis

Quantification of CTB-Pins immunoblots was accomplished using a CTB standard protein (Sigma) at concentrations 0f 25, 50, 100 and 150ng. Volumes of .625 ul decreasing serially for 4 samples were evaluated. Using Alphaimager and Alphaease FC software, the levels of CTB-Pins were quantified.

Chlorophyll extraction

Fresh tissue was ground in an autoclaved mortar and pestle in a methanol/acetic acid mixture until no major particles remained in solution. This extraction was then serially diluted and the absorbance was read at both 650 and 640 which gave the levels of Chlorophyll A and Chlorophyll B. The absorbance was related to a chlorophyll level based on a constant value of define chlorophyll absorbance.

Simulated Digestion assay

Simulated Gastric Fluid (SGF; 0.2% NaCl, 0.7%HCl, 0.32%Pepsin, pH1.2, 37°C) was prepared, and either 1ug of purified PA or plant derived PA was subjected to a 200 ul digestion volume that remained at 37°C for a determined period. Quenching of digestion occurred by Tris/Protease inhibitor cocktail addition, then storage on ice. After digestion, protein extraction occurred via a 1:1 addition of PEB, followed by sonication as previously described. Results were evaluated by loading 50ng of protein for western blotting.

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