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Jack M. Widholm · Jochen Kumlehn · Toshiyuki Nagata
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Commercial Plant-Produced Recombinant Protein Products

Case Studies

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Dedication
In Memory of Michael Horn (1955–2012)

In 2012 the scientific community lost one of its most dedicated scientists in the field of plant cell culture and genetics, Dr. Michael Horn. Dr. Horn succumbed after a short illness to amyotrophic lateral sclerosis (ALS), sometimes referred to as "Lou Gehrig's disease." Dr. Horn spent his career discovering the scientific principles that have been applied to a variety of purposes including plant propagation, somaclonal variation, and recombinant expression of foreign proteins in plants.

Dr. Horn received his B.A. in 1977 and MA in 1980 from the Department of Biology, University of Missouri. He then moved to the Department of Agronomy, University of Illinois, where he received his PhD in 1984 working with Dr. Jack M. Widholm on the "Establishment, Optimization and Characterization of Photoautotrophic Soybean Suspension Cultures." He continued to pursue his research passion throughout his career, albeit in many different companies due to the flux in the biotechnology industry. These included Ciba-Geigy, Plant Genetics/ CalGene, Agrigenetics/Mycogen, ProdiGene, Applied Biotechnology Institute, Targeted Growth, and Cibus.

Dr. Horn used his technical skill to develop improved methodology for cell cultures, transformation, and gene expression. He then applied these methods to a number of different crops (e.g., rice, peanuts, corn, tobacco, and orchard grass) and to a number of different projects (improved agronomic traits, improved food quality, and production of pharmaceutical and industrial proteins). While the scientific community, along with his family, will miss his personal and professional contributions, he will not be forgotten. Many of the chapters in this book describe work that is either a direct or indirect result of Dr. Horn's contributions.

Throughout his career in industry, he kept engaged in scholarly activities including being an author or inventor on over 35 publications and patents. He was involved in several professional organizations, most

notably in the Society for In Vitro Biology (SIVB; formerly TCA) where he was editor of ExPlants and secretary and president of the plant section. He was also Publications Committee Chair and as such brought Springer in as publisher of In Vitro Plant and In Vitro Animal.

Dr. Horn will be remembered by many for his scientific achievements but those that had the opportunity to know him personally will undoubtedly have special recollections of his presence. He continually brought up new ideas to pursue but was extremely tolerant to others when they did not agree with his analysis. He worked steadily throughout any project always trying to make it succeed despite the many roadblocks that are frequently encountered in new fields of research. In short, he behaved as the rest of us strive to act, as model scientists.

In addition to his professional activities he never abandoned his responsibility as a husband, parent, and grandparent. These personal connections always kept him well grounded to the most important aspects in his life. While these gave him a considerable amount of enjoyment, they also provided him with support for his professional obligations. This was most apparent from the tireless work of his wife, Patricia. She provided support for him over his entire career but her dedication was most obvious after he was diagnosed with ALS. While his mind was as sharp as ever, he could not physically do many simple tasks. Patricia therefore provided him with the physical support he needed including driving

him to work, reading, translating, and writing letters when he could no longer physically do these himself.

During this entire time we knew him, he was never without hope including in the later stages of his disease. Even toward the end of his life, after he had lost all mobility and much of his speech, he accepted his limitations but continued to do whatever he could for the family and the scientific community while never losing his sense of humor. He has left a legacy for many of us to emulate as an outstanding role model to balance both a professional career and a personal life. We dedicate this book to his memory.

Preface

Large-scale protein production has come a long way with the onset of recombinant DNA technology in the 1980s. Initially microbes, such as bacteria and yeast, were the choice of host used to produce commercially important proteins; their short generation time and growth to high densities in bio-fermenters were valuable traits. As technology became more sophisticated, other hosts such as cell lines, animals, and plants were explored. Plants lagged behind most other systems primarily because initial biotechnical work focused on agricultural improvement to crops rather than their use for the expression of novel products.

Attention has since turned to using plants as hosts to produce commercially important proteins. Many reviews have been written about the theoretical aspects of this topic but the present volume is focused on commercial successes: case studies of projects that have commercial potential or products that have already been commercialized, illustrating the advantages that plants can have over bacterial, fungal, or animal cell culture hosts. These case studies demonstrate the hurdles that must be overcome and the benefits of using plants to produce industrial and pharmaceutical proteins as well as vaccine antigens. It is predicted that plant protein production is the beginning of a new paradigm for the commercial production of proteins that over the next decade will expand dramatically.

The commercialization of plant-produced proteins has progressed slowly over the past 15 years since the first introduction of a commercial product demonstrating feasibility. Many factors have contributed to this slow progress, but, in brief, the technology was not robust and predictable in the early stages to compete on a strictly cost basis with other existing platforms, and there was little motivation to fund technology improvements to a system that was considered a threat to existing platforms. In the last several years, however, the advantages of plant production systems beyond the unit costs are enabling the acceptance of the technology. The clear front-runner is the move into an animal-free source of proteins for cell cultures. This may soon be followed by an animal-free source of therapeutics, a rapid system for the production of parenteral vaccines, orally delivered vaccines, and industrial enzymes that can only be produced on the scale that a plant system can provide. The advantages of plant-produced proteins beyond the unit cost are the

key to the initial commercialization. In the longer term as the technology becomes more engrained into the industry, this approach can be used for a variety of other proteins where plants can compete on unit cost as well.

In this volume, the focus is on products from plants that have either been commercialized or that are near commercialization. We have chosen protein products that illustrate the promise of the system, for example, highly purified proteins free of concerns over animal pathogen contaminants, directly delivered proteins such as orally delivered vaccines, or minimally processed industrial products.

This book is divided into four parts. The first part on *highly purified proteins* describes trailblazing technologies that are effective for the production of proteins at commercial production levels, at pharmacological and research-grade purities. Some of these proteins are toxic to cells when expressed at even moderately high levels, so they represent a major advance in strategies for the production of proteins that may interfere with normal cellular pathways. These strategies may be modified for use in non-plant systems.

The second part on *vaccines* examines strategies for administration of plant-produced antigens through oral and parenteral routes and for human and veterinary applications. The failure of straightforward approaches to vaccine production for pathogens that show antigenic drift has been addressed by the use of novel strategies such as transmission blocking vaccines, and these strategies may be extrapolated to other vector-transmitted diseases. Antigens that are presented in a structural form that resembles the pathogen are also examined. For veterinary application, vaccines effective for use in domestic herds and wild animals are examined. Some of the outcomes pursued are effectiveness, rapid production, cost-effectiveness, and ease of administration.

The third part on *industrial proteins* evaluates the production of proteins that have applications in the paper and food industries. A unique feature of these proteins is that they can perform their purpose without purification to homogeneity. Cellulase enzymes are effective for conversion of cellulose to biofuels but also for making wood amenable for conversion to paper pulp without the use of environmentally unsafe chemicals. Thus, the indirect effects of the use of these enzymes are also beneficial.

The final part on *future directions* examines the benefit of plants as hosts and reviews some of the possible applications and the regulatory and public perspectives with regard to their use.

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Contents

Chapter 1
Introduction: Plant-Produced Protein Products

Elizabeth E. Hood and Paul Christou

1.1 A Short History of Recombinant Protein Production in Plants

Recombinant protein production in plants encompasses vaccines, pharmaceuticals, and industrial proteins. Within each of these categories are numerous products and host systems with applications to multiple diseases and industrial processes. This industry requires gene transfer from other organisms into plants and allows the plants to overproduce the proteins for the desired application.

Several companies and university laboratories have had programs in plant expression of proteins over the past two decades. The plant biotechnology companies that are focused on production of those proteins are listed in Table 1.1. Significant effort has gone into developing these new products using several plant systems. The choice of system depends on many factors including the type of protein, the technology utilized, the platform of the company, and the funding source (Howard and Hood 2005). Several of these companies are still functional, and others have closed but reemerged as new entities.

1.2 Advantages of Using Plants

Compared to animal and microbial systems, the advantages of using plants for protein production are numerous. For example, plants do not harbor animal pathogens, which is particularly advantageous for pharmaceuticals and vaccines

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Table 1.1 Protein production companies, crops, and product foci over the last three decades

Company	Crop	Main products	Status	Comments
ProdiGene	Maize	Pharma, vaccines, enzymes	Development	Inactive
EpiCyte	Maize/tobacco	Antibodies	Development	Out of business
SemBioSys	Safflower	Pharma, vaccines	Clinical trials	Out of business
Meristem	Maize/tobacco	Lipase, lactoferrin	Development	Out of business
Crop Tech	Tobacco	Enzymes for ERT	Licensed to Protalix and Pfizer	Out of business
Biolex	Lemna	Pharmaceuticals	Research only	Out of business-some products in development by Synthon
Medicago	Transient tobacco	Pharma, vaccines	In production	Company sold to Mitsubishi
Planet Biotechnology	Stable tobacco	Anthrax antitoxin	Successfully protects animals in trials	Planning clinical trials
Ventria	Rice	Blood proteins and therapeutics	Selling research products	Human clinical trials in process
Icon Genetics	Transient tobacco	Biotherapeutics and monoclonal antibodies	Clinical trials in process	Acquired by Nomad
Syngenta	Maize	Amylase—enolase for corn ethanol	In production	First industrial output trait deregulated
Applied Biotechnology Institute	Maize	Cellulases, HepB vaccine, brazzein	Development	Licensed ProdiGene technology
Infinite Enzymes	Maize	Cellulases	Reagent sales	Other enzymes in pipeline
BioStrategies	Transient tobacco	Pharma enzymes for ERT	Development	SBIR funded
Caliber Biotherapeutics	Transient tobacco	Vaccines and monoclonal antibodies	Preclinical trials	Production with G-Con pods
Fraunhofer MBC USA	Transient tobacco	H1N1 and malaria vaccines	Preclinical and clinical trials	Nonprofit organization
Kentucky BioProcessing (formerly LSBC)	Transient tobacco	Aprotinin, vaccines, pharmaceutical	Used in research and cell culture	In production
Mapp Biopharmaceutical	Transient tobacco	Monoclonal antibodies	Preclinical trials	Made by Kentucky BioProcessing

Protalix	Carrot cell culture	Glucocerebrosidase	In production for Gaucher's syndrome	Primarily Israeli market
ORF Genetics, Iceland	Barley	Human growth hormone, cytokines	Diagnostics, research, cosmetics	In production from seed
Dow Agrosciences	Cell culture	Newcastle disease viral vaccine	Approved	Unknown if company is selling
Inserogen	Tobacco	Vaccines and biotherapeutics	Development	Start-up

ERT enzyme replacement therapy, *LSBC* Large Scale Biology Corporation

(Ramessar et al. 2008; Sabalza et al. 2011). Pathogen-free pharmaceuticals are desirable whether delivered orally or through injections. Thus, the plant host can be a food crop, such as corn, canola, or rice, or a nonfood crop, such as tobacco.

Because several of the plant hosts are food plants, oral delivery of the proteins for therapeutic purposes is possible. Oral delivery has been demonstrated for potato (Tacket et al. 1998), corn (Lamphear et al. 2004; Hayden et al. 2012), and banana (Mason et al. 2002). In each case, the integrity of the protein must be ensured through the formulation process, e.g., extrusion or cooking. If raw, the plant host must be edible without processing, such as a fruit or vegetable. In contrast to injected pharmaceuticals, the cold chain may not be required to transport these orally delivered products to the target population, which is particularly useful when serving developing countries. This is a distinct advantage for plant systems—high product stability at ambient temperatures.

Direct addition of the proteins in their host tissue may be possible without the need for purification. This can be an advantage for pharmaceuticals as well as industrial proteins and enzymes. The less processing required for a formulation, the more cost-effective the manufacturing. Thus, direct addition of the plant part containing the enzyme of interest saves money on production and increases the margin for the producer. Direct addition would be particularly useful for industrial enzymes that accumulate in dry seed, such as corn, where stability is ensured in the seed until such time as it is used (Howard et al. 2011).

An additional advantage is when current agricultural crops are used as plant hosts; their production and processing are well established and usually inexpensive. As an example, corn requires few inputs other than nitrogen if grown in the corn belt. Dry mill processing is very well established on a volume basis, and every fraction of the whole or milled corn has a market. If value can be added to one of the lower value coproducts, for example, by putting a high-value protein in the germ (Hood et al. 2007), then an advantage is gained in increasing the value of this coproduct of the corn-to-ethanol industry.

Scaling up production of proteins from crops is also advantageous over animal or microbial systems. For crops, scale-up involves planting and harvesting more acres and does not require additional capital investment in physical infrastructure. The only capital investment involves planting and harvesting equipment, which, although somewhat expensive, does not require the level of investment required for scaling up microbial or animal systems. Thus, high-volume production can be achieved relatively easily.

1.3 Issues for Commercialization

Intellectual property for the specific gene and its expression in a plant host is only one part of the legal landscape for commercializing products using the plant production platform. Plant-enabling methods have been developed over many years with many companies and university laboratories participating in the

platform. Thus, a plethora of patents surround the technology and are often barriers to entry for commercialization of products from genetically engineered plants. During the development of potential products, it is critical to be aware of the technology pieces that are utilized to ensure freedom to operate on the pieces. Licenses for technology can sometimes burden the developer with high royalty fees, pushing the products' costs to a price greater than they are worth.

1.3.1 Regulatory Issues and Public Acceptance

1.3.1.1 Europe

The European Food Safety Authority (EFSA) is a European Union (EU) agency mandated to evaluate the risks of all transgenic crops based on scientific evidence. This evidence is evaluated by a panel of experts, and testing is carried out at an EU reference laboratory. As such, EFSA is best placed to advise individual Member States and the EU as a whole on safety issues (Sabalza et al. 2011). EU legislation for the approval of GE crops (Directive 2008/27/EC and Regulation EC 1829/2003) is the most onerous and restrictive in the world. Regulatory compliance for a new crop with first-generation simple agronomic traits can cost up to €11 million (~US$15 million) and requires a dedicated legal team working for many years (Kalaitzandonakes et al. 2007).

The EU regulatory approach is precautionary, process-based, and includes mandatory labeling and traceability requirements (Ramessar et al. 2008). The approach has been described in detail in a recent review (Sparrow et al. 2013). Briefly, EU legislation is adopted through a system of interactions between the three main EU institutions: the European Parliament, the Council of the European Union, and the European Commission (Sparrow et al. 2013). The EFSA published guidance notes in 2009 on the risk assessment of genetically modified plants used for nonfood or non-feed purposes (EFSAPanel 2009) including molecular pharming applications. The European Medicines Agency (EMEA) that oversees the assessment of biopharmaceuticals and vaccines published guidance notes in 2006 on the "quality of biological active substances produced by stable transgene expression in higher plants" (EMEA 2008), which looks at such issues.

More recently a further requirement was imposed on all transgenic plants, including those for molecular pharming applications. The European Commission mandated a compulsory 90-day animal feeding trial and, to make matters even more complicated, is considering extending that to a 2-year trial based on the now-discredited article by Seralini et al. (Seralini et al. 2012; Arjó et al. 2013). The scientific community as well as regulators themselves questioned the validity of such whole food-based animal trials (Kuiper et al. 2013).

Once authorization has been received, farmers must ensure that they comply with the conditions laid down by the authorities in their Member State and/or local region, often finding that illegal national or regional bans on GM agriculture have

been imposed. Farmers must abide by the coexistence measures that have been implemented in each Member State or region, and the complexity of these regulations and their strict implementation often means that it is impossible to comply. The four major obstacles to GM agriculture in the EU post-authorization are:

1. Public field registers showing the location of commercially grown GM crops are compulsory in almost all Member States and tend to discourage farmers from adopting GM agriculture because of the threat of vandalism by activists.
2. Six Member States use a "safeguard clause" nominally based on environmental or health concerns, to implement national cultivation bans for approved GM crops (Austria, France, Germany, Greece, Luxemburg, and Hungary).
3. Stringent coexistence measures have been implemented in Belgium, the Czech Republic, Germany, Hungary, Portugal, Romania, and Slovakia, which make it impossible to grow GM crops without risking litigation from the surrounding farms.
4. The negative publicity surrounding GM agriculture in Europe, which means farmers are ostracized and intimidated directly or indirectly.

The public in Europe has adopted a predominantly anti-GM stance, which is fueled by politicians and media eager to exploit public sentiment. This vicious cycle also shows no sign of going away any time soon (Farre et al. 2011). As discussed above the rules governing the commercial cultivation of GM crops in Europe are obstructive and arbitrary, making it virtually impossible for a farmer to make an independent decision to adopt the technology on his/her land even if the crop in question has been approved (Ramessar et al. 2010).

Across Europe the political viewpoint of cultivating GM crops is far from harmonious, with a number of Member States banning such cultivation (http://www.greenbiotech.org) (Ramessar et al. 2008, 2009, 2010; Sabalza et al. 2011). Given the state of play surrounding the cultivation of agricultural GM crops, it is unlikely that we will see a pharmaceutical crop grown commercially in Europe any time soon (Masip et al. 2013; Sparrow et al. 2013).

1.3.1.2 United States

Regulations in the USA for transgenic plants are set by the United States Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA). The regulatory framework is complex and expensive with a lack of standardization for data collection and analysis (Hood et al. 2012). The framework is somewhat coordinated in that each agency is responsible for specific types of approvals—USDA for plant pests, FDA for food and feed issues, and EPA for pesticides, although sometimes the lines overlap or are blurred. A recent review describes the legislation and several case studies that apply the standards as they currently stand in the USA (Sparrow et al. 2013). To facilitate the process, particularly for small and specialty crop developers, a basic road map

should be created from which a specific regulatory path can be planned and implemented (Hood et al. 2012).

Public acceptance in the USA is much less of an issue than in Europe. Although anti-GMO groups are active in the USA, their impact has waned over the years. The success of genetically engineered crops has been good, showing higher yields and fewer pesticide or herbicide inputs. The vast majority of corn and soybeans in the USA are produced from GE crops and occur in many processed foods. Thus, even though some resistance occurs against GE plants in the press, the basic fact is that most citizens are consuming GE foods on a daily basis without incident. Indeed, each of the crops was subjected to a vast array of safety studies that were reviewed not only by the USDA APHIS but also by the FDA to ensure human safety. Miller (2011, 2012) published some editorial opinion pieces recently on the status of GE crops worldwide and received a great deal of criticism. However, the facts are correct and supported by such groups as the Grocery Manufacturers Association (http://www.gmaonline.org/news-events/newsroom/gma-commends-ama-action-in-support-of-continued-use-of-genetically-engineer/).

1.4 The Case Studies

Several reviews of plant-produced proteins have been written over the last several years (Fischer et al. 2004; Stoger et al. 2005; Streatfield 2007; Daniell et al. 2009; Egelkrout et al. 2012). Each of these reviews describes issues concerning expression, different product categories, and advantages of different plant systems. Plant biotechnology and gene transfer have been practiced as a technology since the early 1980s, and the vast majority of products commercialized have been input traits that assist with production, e.g., insect and herbicide resistance (Castle et al. 2006; Fraley 2009).

In this volume, the focus is on products from plants that either have been commercialized or that are near commercialization. We have chosen protein products that illustrate the promise of the system, for example, highly purified proteins without concerns over animal pathogen contaminants and directly delivered proteins—orally delivered vaccines or minimally processed industrial products. The promise of plant-made recombinant proteins was first realized in 1997 with the introduction of avidin and β-glucuronidase. Recently, pharmaceuticals (PMP) and vaccines as well as industrial proteins (PMIP) have just recently been consummated with the introduction of Syngenta's Enogen corn that contains amylase for the starch to ethanol application (Pollack 2011) and Protalix and Pfizer's glucocerebrosidase for enzyme replacement therapy (Aviezer et al. 2009; Ratner 2010). The products described in these chapters do not represent all the work that has been done in transgenic plants but do represent several that have been moved into or near commercialization.

Hood and Howard describe development of avidin in corn seed, originally transformed in as a potential candidate for insect resistance. Although the insect resistance trait was not commercialized, avidin was subsequently purified from seed and sold. This product was a key achievement for the plant manufacturing industry as the first protein sold from transgenic plants (Hood et al. 1997) (Sigma Chemical Co. A8706) and set the stage for this platform (Chap. 2). Although the avidin market is small, its importance cannot be overstated since it was the demonstration product for the technology. The main application of this protein is as a research reagent that allowed quick market entry.

Other types of products such as vaccines and pharmaceuticals were also in development concurrently but had much longer timelines for market entry. Fischer et al. (Chap. 3) describe multiple therapeutics that include antibodies for several applications manufactured in plant production systems. These therapeutics are produced by a number of different platform technologies, and the issues for their commercialization are discussed in the context of these new products.

Krishnan and Woodard (Chap. 4) describe the development of recombinant trypsin from the maize seed production system. This product is sold under the trade name TrypZean™ and is currently used for research and for processing of therapeutic proteins. One of the largest applications of trypsin is the maturation of recombinant insulin, and the plant-derived protein could be a great improvement in this process since it is animal product-free and would not pose threats to the drug's use.

Aprotinin is manufactured in the transient tobacco system using an engineered tobacco mosaic virus vector (Chap. 5). It has major applications in surgery as a preventative for perioperative blood loss. The plant-made aprotinin is currently not approved for human use but has applications as a protease inhibitor in cell culture.

Vaccines are particularly well suited for plant production because of broad application and current need for a cold chain. Vaccines against a number of viruses have been developed using plant expression systems. Pandemic flus can threaten world health quickly and catastrophically. In order to address the need for rapid development of vaccines against urgent threats, Medicago Inc. established a platform technology that addressed surge capacity, speed, adaptability, and affordable cost per dose. The company developed a vaccine against the H1N1 flu virus in a transient tobacco expression system (Chap. 6) and showed efficacy in Phase I and Phase II clinical trials. Further development of the vaccine will be performed by Mitsubishi who recently acquired Medicago.

Malarial vaccines are extremely useful in tropical climates where mosquitoes are abundant. Streatfield et al. (Chap. 7) discuss the transient tobacco transformation system for the production of such a vaccine against the malarial parasite that is spread by the mosquito vector. Subunit vaccines using individual proteins have been difficult to develop because of the difficulty in expressing the individual antigens. The plant system has been particularly useful in this regard.

Transmissible gastroenteritis virus (TGEV) is a common pathogen of swine and is particularly dangerous to newborn piglets. Rajan (Chap. 8) describes the development of a subunit vaccine in corn seed that shows efficacy against the disease, particularly when delivered orally either through feed or colostrum from the sows. Although this highly efficacious and easily administered vaccine is available, it has not been adopted by the swine industry.

Many species and strains of rabies virus are known, posing a threat to human health worldwide, but particularly in developing countries. Loza-Rubio and Rojas-Anaya (Chap. 9) discuss the issues surrounding the development of a rabies vaccine based on the G-protein expressed in either corn seed or carrot roots. Both sources of the protein provided protection against the rabies virus in superinfected animals. These results are promising for the future of inoculation of wild animal populations to lower the load of infective viruses.

Newcastle disease virus is highly infective in avian species and can devastate poultry production in many countries. Gomez-Lim (Chap. 10) describes the development of plant-based vaccines against this virus using the corn/sorghum seed system for oral delivery or the tobacco system for injectable delivery. The ease of delivery of oral products would seem the preferred route and various issues to be overcome for this application are discussed.

Although several injectable vaccines for hepatitis B virus (HepB) are available, infection with this virus remains a world health problem. Hayden discusses the development and feeding trials of a plant-made oral vaccine from corn grain (Chap. 11). Oral vaccines have many advantages in that they have higher rates of dose compliance among susceptible populations. Using formulations of corn germ derived from transgenic plants expressing the S antigen, successful production of mucosal protective antibodies was achieved in mice.

Hood and Requesens (Chap. 12) describe the development of the industrial enzymes endo- and exo-cellulase in maize grain. These enzymes have applications in research, pulp processing, and biomass conversion. Early markets have been addressed with these products, and production lines have been established.

Finally, the sweet protein brazzein has been produced in maize grain. Fake and Howard (Chap. 13) describe the applications of this protein in various food-related industries and the effort to interest food companies in its use. Because the protein is a natural sweetener from an African fruit, it would be a logical substitution for such artificial sweeteners as acesulfame potassium or aspartame, particularly also because brazzein is about 1,000 times sweeter than sugar.

In the final chapter, the future of the plant-based production industry is discussed. Prospects are promising, but the major commercialization barrier is still overcoming the regulatory hurdles. Drs. Howard and Hood are pleased to present these case studies of plant-made proteins as a tribute to our colleague, Dr. Michael Horn.

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Part I
Highly Purified Proteins

Chapter 2
Commercial Plant-Produced Recombinant Avidin

Elizabeth E. Hood and John A. Howard

2.1 Introduction to the Protein Product

Chicken egg white avidin was the first recombinant protein product manufactured for sale in a transgenic plant. Prior to its commercialization, there were many questions as to the validity of using plants as a platform to produce recombinant proteins: doubts were raised as to the ability of plants to express animal or microbial proteins, the ability to obtain proper processing and glycosylation, and the ability to extract and purify these proteins in an economical manner. Therefore, while avidin has modest economic value, it served as the model to launch this approach for a number of other recombinant proteins.

Avidin (C.A.S.: 1405-69-2) is a glycoprotein found in avian, reptilian, and amphibian eggs and is used commercially as a diagnostic reagent. It was first isolated from chicken egg white and named "avidin" in the 1940s (Thompson et al. 1941). The protein avidin comprises four identical subunits, each 128 amino acids long, the amino acid sequence of which was published in 1971 (DeLange and Huang 1971). The carbohydrate moiety is composed of four glucosamine and five mannose residues and is attached to Asn-17 of each subunit (DeLange and Huang 1971). The cDNA of the chicken oviduct *avidin* gene was identified (Gope et al. 1987) and a genomic clone was isolated (Keinanen et al. 1988). They (Keinanen et al. 1988) also reported on a family of closely related *avidin* genes from chicken.

Avidin binds the vitamin biotin with high affinity. Each of the four subunits in the homotetramer binds one biotin molecule. The dissociation constant of the avidin–biotin complex was determined to be 10^{-15} (Green 1963), exhibiting the

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highest known affinity in nature between a ligand and a protein (Livnah et al. 1993). The binding of avidin to biotin is responsible for its commercial value, since it allows for detection of proteins and nucleic acid molecules incorporating biotin. Avidin or avidin subunits can also be used for affinity purification of biotinylated molecules (Berger and Wood 1975; Green and Toms 1973). In nature biotin functions as a cofactor with many enzymes in vivo. Because avidin binds strongly to biotin, it can act as a defense agent against microbial pathogens that are sensitive to biotin levels (Wallen et al. 1995). A second biotin-binding protein is bacterial streptavidin. Although these two proteins show similar activity and tertiary structure, their amino acid sequences are only 30 % identical and are likely not derived from the same ancestral source (Laitinen et al. 2006).

Scientists at Pioneer Hi-Bred International noticed that avidin could inhibit growth in some insects by interfering with their digestion. Transgenic maize plants expressing the chicken *avidin* gene were generated to test it as an insecticidal reagent incorporated into maize leaves and roots (Hood et al. 1997). This observation was later followed up and shown to be very effective to prevent postharvest insect damage while not interfering with metabolism in mammals (Kramer et al. 2000). The transgenic maize plants had a secondary phenotype in that they could confer male sterility and have been suggested as a containment mechanism for transgenic traits in the field (Albertsen et al. 1999).

The primary source for commercial production of avidin is chicken egg white, although the recombinant form is also available (Sigma Chemical Co. A8706). The manufacture of purified avidin protein using a plant source as an alternative to eggs provides benefits such as the absence of animal viruses. Plant-produced avidin provided answers to many of the basic questions about plant-expressed proteins and what is critical for commercialization, providing conditions that are in use today.

2.2 Description of the Systems Used to Produce the Protein

2.2.1 Theoretical Advantages of the Plant Process over Other Technologies

Avidin is usually purified from egg whites (http://www.mastbio.co.kr/root/product/life/ps/gradiflow/pdf/MB-10-Puri-HighlyBasicProteinsAvidinandLysozyme.pdf), where it is present at a concentration of approximately 1.5 mg per egg. More recently, biologically active recombinant isoforms have been produced in several expression systems, including *Escherichia coli* (Airenne et al. 1994), *Picchia* (Zocchi et al. 2003), and baculovirus-infected cells (Airenne et al. 1997). A huge number of variants of avidin have been produced that have applications in various diagnostic and purification kits (Laitinen et al. 2006).

The advantages of a plant recombinant system over the others currently used are that: (1) scale-up is more economical in a plant system due to less expensive substrates (corn grain versus eggs) and greater biomass availability, (2) co-purification of animal pathogens is avoided in a plant system, and (3) if expression is directed to seed, it provides a natural storage system for long duration without degradation.

2.2.2 Past Efforts in Plants

A number of laboratories have experimented with expressing avidin in plants, primarily for its insecticidal properties (Murray et al. 2002; Lichtfouse et al. 2010; Burgess et al. 2002; Markwick et al. 2003; Murray et al. 2010; Masarik et al. 2003). In many cases, the transgenic plants were insect resistant reaching the goal of the project.

2.2.3 Bench Marks of What Is/Was Needed to Commercialize the Product in This System

Most of the initial work with avidin expression in different plants was not designed to overproduce the protein for purification and sale. The maize seed production system, on the other hand, was suitable for the production of the protein for sale as a purified or partially purified product primarily for use in diagnostic kits. High-level expression is required for cost-effective production in the plant system to meet commercial targets. Assuming that the competitive production system is from egg whites, one dozen eggs would produce about 18 mg of avidin for a cost of about $2 for the raw materials. Eighteen mg of recombinant protein from corn seed expressing the protein at 1 % of total soluble protein would require approximately 200 g of grain. At today's high price of $7 per bushel (25 kg), this grain would cost ~$0.06. One percent of TSP has been achieved for multiple proteins in corn seed, and avidin levels as high as 40–50 % of TSP in some selected lines have been obtained. Clearly, the corn system offers economic advantages over the egg system as it relates to the cost of raw materials. In addition, higher concentration of avidin in the biomass leads to a lower cost of purification.

Because proteins produced from plants were new to the market, quality assessment of the product had to be performed to understand the impurities in the product and to build a certificate of analysis, a quality control protocol, and a Material Safety Data Sheet (MSDS) for the product. Each of these was developed for this new product for Sigma Aldrich Chemical Co., which is still the vendor for the product. Characteristics of the protein and product are described below.

2.3 Technical Progress

2.3.1 What Was Achieved?

Many technical tools that were sought after in the mid-1990s are the same today for expression of foreign genes in plants. These include use of a strong promoter, use of an intron particularly for monocot expression, recognition of the need for codon usage that is compatible with the host species, avoidance of toxicity, and targeting the protein to specific subcellular locations that induce maximum expression of the protein (Streatfield 2007). Indeed, each of these molecular parameters was utilized for avidin.

Avidin in maize seed was first produced over 16 years ago (Hood et al. 1997). The molecular technology available at the time was much less sophisticated than technology available today. The gene was synthesized with maize codon usage bias and fused with the barley alpha amylase signal sequence (BAASS) (Rogers 1985), also synthesized with maize codons. Each of the genes/fragments was synthesized as short, overlapping, complementary oligonucleotides with restriction enzyme sites engineered onto the ends and ligated after digestion. All movement between cloning vectors was done with restriction enzyme digestion and ligation. The expression cassette with the constitutive maize ubiquitin promoter (Christensen et al. 1992) and the *pinII* terminator (An et al. 1989) was built separately from the herbicide selection vector for co-bombardment of maize callus tissue. Selection was on the herbicide, bialaphos, using the *bar* gene (White et al. 1990) driven by the CaMV 35S promoter. At that time, biolistic transformation was the most efficient way to introduce genes into corn (USP#5,489,520).

2.3.2 What Expected or Unexpected Hurdles Were Overcome to Reach the Target?

Transgenic events that were resistant to bialaphos and contained the avidin gene as identified by PCR were recovered from transformations. Plants were regenerated from these events; they produced ears in a greenhouse and were pollinated with a proprietary inbred line (Pioneer Hi-Bred PHN46). The highest expressing event was screened by DNA blot hybridization for copy number and insertion sites (Hood et al. 1997). It appeared that three to five insertions were present in this event for both the *avidin* and *bar* genes. When T1 seed was planted for seed increases, the T2 generation plants were no longer resistant to the herbicide. Thus, another method of screening for the segregating (transgenic versus non-transgenic) plants was required. Initially, PCR was performed to track the presence of the *avidin* gene. Observations of the plants in the field revealed that male sterility was present among them at a high percentage. When the PCR results were compared to the