

Commercialization of Biopharmaceutical and Bioindustrial Proteins from Plants

John A. Howard*

ABSTRACT

Over the past decade many new products have entered the market as the result of biotechnology. In regard to biotechnology tools related to plants, this has mainly been in the form of products that conferred crop improvement traits. The technology developed for crop improvement, however, can be applied to commercialize nonfood products such as those developed by other host systems such as microbial and animal cell culture systems. The first wave of therapeutic proteins is now in clinical trials for both animals and humans. Other nonfood products are now being introduced into the marketplace. This paper examines a few of these representative products to highlight the potential advantages and hurdles that need to be overcome to bring these products to the market. This includes not only the technical barriers but also what is needed for both regulatory and public acceptance.

THERE HAVE BEEN MANY PRODUCTS developed by biotechnology over the past two decades. With respect to plants, however, most of these are not new products but rather existing products with improved traits. For example, insect- and herbicide-resistance genes have been engineered into commodity crops (Babu et al., 2003; Gressel, 1993). Transgenic plants carry these new traits, which have very beneficial effects for the grower. Seed companies sell these new traits as part of their existing product line. The end user, however, sees no difference in the commodity crop produced.

This situation is in contrast to genes engineered into other organisms such as bacteria, yeast, or animal cells. In these instances, new genes are transformed into the host to produce entirely new products such as pharmaceuticals or industrial proteins. Rarely in these cases are genes introduced into the host organisms to improve the characteristics of the host organism itself for commercial purposes.

As a result of genetic engineering for new traits that has been applied to plants, the technology is available for plants to provide a host system for new products (Hood and Howard, 2002; Cunningham and Porter, 1998). Expanded use of genetic engineering in plants will allow the development of new products that would have been limited or unattainable by alternative host production systems. Currently, the most economically and technically viable class of products are obtained from engineering genes to produce protein products. This has resulted in a \$40-billion industry of new therapeutics and industrial enzymes (Van Arnum, 2003). While this has been extremely useful and lucrative, the host systems used today can still be limiting. Microbial systems are the most popular and cost effective but are limiting because of downstream processing. Yeast can

cause hyperglycosylation, and bacteria do not glycosylate. Animal cell cultures are better hosts for downstream processing. However, the cost of animal cell culture systems is orders of magnitude higher than their microbial counterparts. There is the potential to use transgenic animals in the future as a host. These will undoubtedly prove useful; however, one disadvantage is that the protein product will be derived from an animal source. This is significant because there is a mandate from many pharmaceutical companies to remove all animal source materials from their products. Therefore, an opportunity exists for plant systems to increase the safety margin of these products since plants do not harbor human pathogens. Plants may also provide a lower cost of goods and a greater convenience (Kusnadi et al., 1997). Increased use of plants for protein production will result in new products that can help improve our quality of life as well as provide safe alternatives to environmental risks.

Product Examples

While potential benefits of this new technology are great, there are many hurdles to overcome to bring these products to market. These include technical, economic, and regulatory challenges. In addition, public awareness of genetic engineering must be increased to gain acceptance. Technical challenges are numerous but can be simplified for the purpose of this paper by asking the following questions. Can plants make functionally equivalent proteins? Can these proteins be purified from the plants in its physiological active form? Can this be done to meet commercially important targets?

These technical questions were examined several years ago with a proof-of-principle product, avidin. Avidin is a protein normally found in chicken egg whites and binds biotin (DeLange and Huang, 1971). It performs as a valuable reagent in many diagnostic tests for human health. The objective was to use avidin to demonstrate that a product could overcome many technical challenges facing the technology to bring a product to market.

Avidin was introduced into plants by particle gun bombardment, and commercially economic levels of expression were obtained under the control of a constitutive promoter (Hood et al., 1997). The protein made in plants was functionally equivalent to that obtained from chicken egg whites, its native source. More importantly, this example demonstrated that the raw material cost was a 100 times less than that of eggs, already an inexpensive source of protein. The amount of biomass needed for purification was 10 times less than that needed of eggs resulting in reduced processing costs. The grain could be stored for years without any loss of activity making it convenient for storage and transport. In addition, the threat of contamination to workers due to salmonella was eliminated by removing the animal source. Avidin clearly demonstrated the feasibility of

5819 Stallion Ridge, College Station, TX 77845. Received 9 Dec. 2003. Symposia. *Corresponding author (jhoward999@earthlink.net).

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the technology, and the plant-derived protein is currently being sold through ProdiGene (College Station, TX) or Sigma Chemical Company (St. Louis, MO).

The most important factor in making protein products commercially viable is the expression and accumulation of proteins in the host. While the avidin market is relatively small, this example provides an opportunity to investigate what is needed to improve protein accumulation in plants. Steps including transcription, translation, intracellular localization, tissue specificity, copy number, and metabolism may need to be optimized in different ways for avidin as well as other proteins. These various parameters, as illustrated in Fig. 1, can each have a dramatic influence on specific proteins by themselves or in combination with each others. While it is difficult to predict at this time the most critical component for specific proteins, as more information is accumulated, better predictions will be possible.

The accumulation of avidin in the grain increased dramatically over the years as shown in Fig. 2. This was due to a combination of the various parameters including promoters, targeting sequences, and germ-plasm selection. The expression level in corn (*Zea mays* L.) has been confirmed to be over 25% of the total soluble protein or nearly 2 g avidin/kg corn grain (Masarik et al., 2003) by independent groups using different methods of analysis. Since most of the avidin is in the germ portion in this example, up to a 10-fold enrichment may be possible upon separating the germ from the endosperm by standard mechanical methods. This would then bring the level to over 10 g avidin/kg dry of corn matter placing this in the target range for polymers as well.

Figure 2 also shows an approximation of what level of expression is beneficial to achieve economic feasibility for different types of products. Specialty protein products used as fine chemicals could be economically feasible when expressed as low as 10 mg/kg of the dry weight in a crop such as corn. Most pharmaceuticals would need to be expressed at ≥ 100 mg/kg of plant tissue (Evangelista et al., 1998). The target for industrial enzymes is 1 g/kg, and for polymers, the target is yet higher. These values represent lower limits, and for all cases higher expression is desired.

The expression of specific proteins will vary; some having higher expression levels than avidin and some much lower. This is true for other recombinant production systems as well as for plants. The avidin example, however, does demonstrate that at least the corn system and most likely other plant systems have the capability to achieve expression levels to meet the economic targets for a variety of products.

With avidin demonstrating proof of principle, attention was focused on another protein, trypsin. Trypsin is a protease usually derived from its native source of bovine or porcine pancreas. It is used in numerous applications including food processing, as a digestive aid, in detergents, as a research reagent and as a cell-culture reagent (Saunders and Wormsley, 1975). The most relevant application for this discussion is its use as a bio-processing agent for pharmaceutical proteins. Many recombinant proteins today are produced in bacterial cells, which reduce cost and provide a reliable supply. Unfortunately, since bacteria do not always process proteins in the same manner that is done in their native source, some of these proteins must undergo a protease cleavage step after production in bacteria, and many of them require trypsin. Since trypsin is derived from animal sources, pharmaceutical companies cannot eliminate the use of animal-source tissue in the final product. In addition, trypsin is a difficult protein to express in most hosts since it can degrade the proteins in the host cell. This has presented an opportunity for plants to provide an animal-free source of trypsin provided high expression levels could be achieved.

A gene for trypsinogen linked to a seed specific promoter was engineered into corn using *Agrobacterium*. Plants were then selected for high expression of the protein. Seed was increased, a crop was grown, grain was harvested, protein extracted, and the trypsin purified (Woodard et al., 2003). The corn-produced trypsin was shown to be functionally equivalent to trypsin derived from bovine pancreas. This represented the first large-scale production of a recombinant protein from plants. Trypsin is now available from ProdiGene or Sigma under the trade name of TrypZean and scale up for large quantities is continuing.

The next example is the therapeutic protein, aproti-

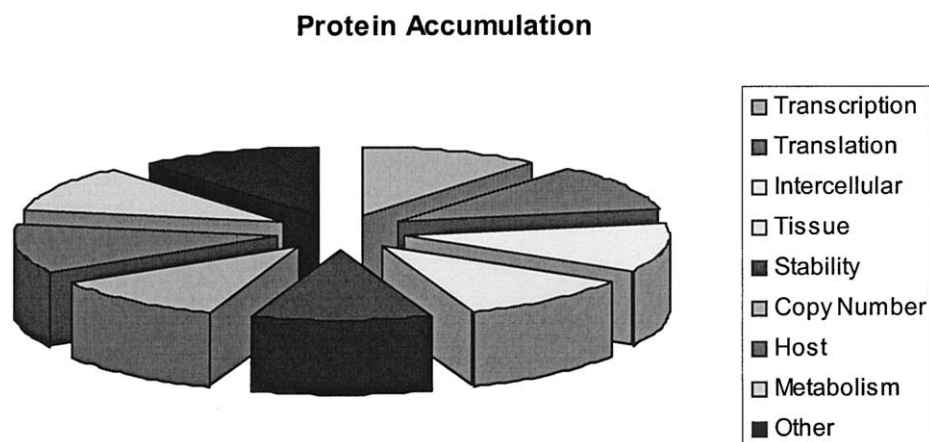


Fig. 1. Parameters involved in obtaining accumulation of recombinant proteins in plants. The different segments each represent areas that can be optimized to increase protein accumulation in the host plant.

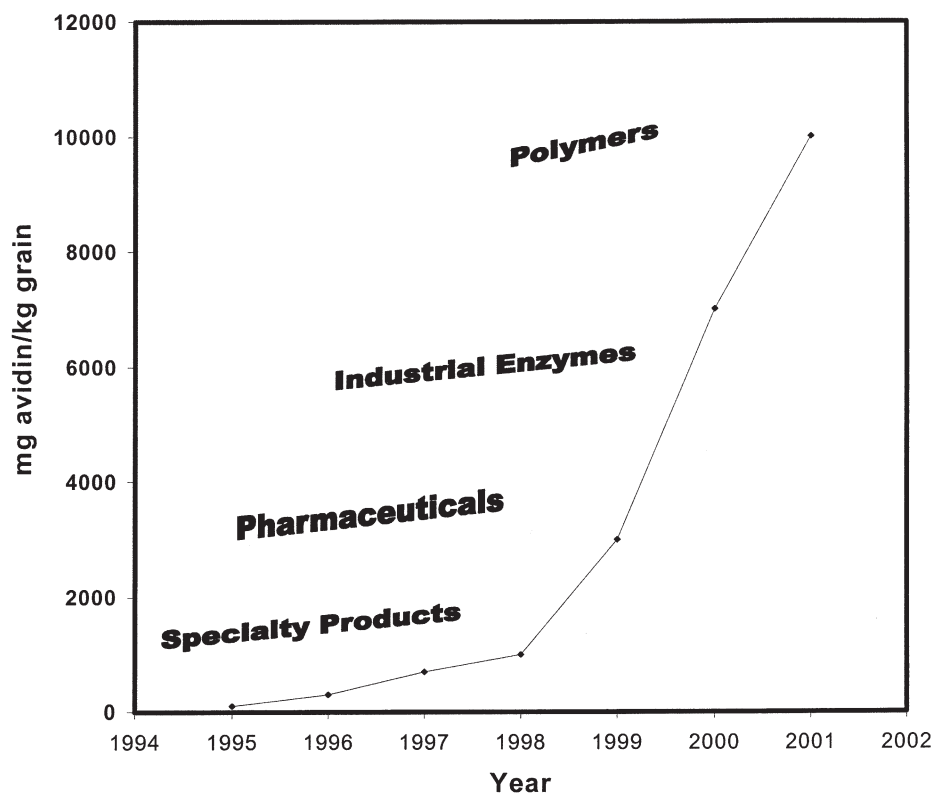


Fig. 2. Technology Curve/Avidin Expression. The expression of avidin was determined each year following a series of technical improvements. The expression represents mg of avidin/kg of grain. The level of accumulation needed for specialty products, pharmaceuticals, industrial enzymes, and polymers is approximated by their position on the graph.

nin. Aprotinin is a protease inhibitor derived from bovine lungs, and it is used in open-heart surgery to prevent blood loss (Beath et al., 2000). It is currently marketed under the name of Trasylol by Bayer Corp. (Pittsburgh, PA). There is some concern over its availability since it is derived from an animal source that must be certified as "Mad-Cow free." The production of aprotinin in plants has the potential to provide an unlimited animal-free source for its therapeutic application. Aprotinin expressed in corn was shown to be chemically and functionally identical to the native source isolated from bovine tissue (Zhong et al., 1999). The chemical equivalency is critical if this drug is to be a generic replacement for the commercial product. Its chemical equivalency should reduce the regulatory hurdles and allow an abbreviated pathway that could greatly reduce its time to market.

The conclusion regarding technical feasibility is that plants can be used to express a number of diverse heterologous proteins. In some cases even when the proteins have been shown to be difficult to express in other systems, plants may allow over-expression. Furthermore, these can be expressed at levels that will have cost advantages and are commercially viable. These proteins are functionally equivalent and, at least in some cases, chemically equivalent allowing them to be used as generic therapeutics thus reducing the fear of human pathogens coming from the source tissue. The only area where plants may not be ideally suited for expression a priori is when the proteins require glycosylation. The carbohydrate sequence used in plant glycosylation is

slightly different than that produced in mammals. In most cases this minor difference does not represent any functional differences in the proteins with one notable exception. Plants, with only one rare exception (Shah et al., 2003), do not add sialic acid onto proteins as part of their glycosylation sequence. In some specific cases for therapeutic proteins, this addition allows for a prolonged clearance time in the blood stream (Rasmussen, 1991). Until this pathway is manipulated to be effective in plants, these proteins must either be produced in animal systems or added in vitro after they are purified from plants.

Direct Delivery

In addition to being a source of raw materials for production, plants offer an opportunity to provide a direct delivery mechanism for many applications. Direct delivery refers to a product that is produced in plants, and the plant tissue is used without purification. This could also apply to producing a protein in plants, and using the plant tissue as a food source, feed source, or feedstock in industrial products.

One example of this approach is plant-based oral vaccines. Oral vaccines have the potential to increase the convenience and compliance of patients. If vaccines are expressed in edible plant tissue, the need to purify the vaccine is eliminated making this a much more economical proposition (Streatfield and Howard, 2003b). This system also has the potential to reduce the dependence on needles and its associated problems, the de-

pendence on the cold chain, concerns over human pathogens, the need for medical assistance in administration, and the overall cost of vaccines.

One example for illustration is that of *Transmissible gastroenteritis virus* (TGEV). TGEV causes a swine disease that affects young pigs and can lead to mortality (Laude et al., 1990). Transgenic corn has been developed containing the spike protein from this virus, the likely candidate for vaccine development. This corn has been fed to pigs, and these animals have been observed eliciting an immune response. Pigs fed the TGEV corn have dramatically increased antibodies titers to the virus compared with control pigs (Streatfield et al., 2001). Experiments have been performed to investigate if this would also give protection from the disease when challenged with live virus. Feeding pigs TGEV corn has resulted in complete protection whereby no disease systems were observed as compared to control pigs fed wild-type corn (Lamphear et al., 2002) as well as lactoimmunity (Lamphear et al., 2004). Similar approaches are under investigation for human vaccines as well (Streatfield and Howard, 2003a, 2003b).

In addition to oral vaccines, direct delivery can also be applied to industrial feedstocks. Many industrial processes that require enzymes also require the addition of plant tissue. One example is the use of enzymes for biomass conversion. In this case, enzymes are needed to convert the plant tissue into suitable substrates for bacteria, which can then be converted into ethanol (Watson, 1988). Corn is already a popular raw material that requires enzymes to convert starch in the grain into ethanol. The corn stover is another potential source of substrate for ethanol but requires a host of additional enzymes to make it suitable for use. One possibility for the future is that corn grain and the stover may be harvested from the same field. The grain itself can then be fractionated into the germ portion and the endosperm that contains most of the starch. The starch from the endosperm can be used for ethanol production as is done today and the germ provides little benefit in this regard. The germ, however, could be the site to over produce the enzymes needed for the conversion of stover into ethanol. In this example, both the enzyme products and the substrates for the final product are produced in the same plant.

Acceptance of New Technology

Scientists working on new cures for diseases and cleaner environmental solutions clearly see the tremendous benefits this technology offers. The general public, however, has developed mixed opinions. While the technology used for human health is accepted by most people, the use of this technology in agriculture has generated concern and controversy (Hallman et al., 2003; Durant, 1991). To address the reasons for this difference, three basic questions can be addressed. Does the public get a choice? Do they understand the technology? Do they see the direct benefits?

In addressing the first question, the public has not been given a choice for genetically modified organism (GMO) food products. A choice for the general public

would involve labeling GMO products that enter the food supply. Instead the public must accept a choice made by others for them. This has led to much controversy about the labeling of GMO products. The net result is that this has left the public skeptical.

Regarding the second question, the public, for the most part, does not understand the technology in producing these new products. In fact, the majority of the population does not understand the underlying science or the safety concerns (Pew Initiative on Food and Biotechnology, 2001). This problem will not be solved soon because it depends on educating generations. The long-term solution requires more science in the school system. In the short term, the best hope is that the public will better understand the risk and benefits of the technology.

Finally, with regard to the third question, the public does not see the direct benefits of GMO plants used for food or feed. The general public is not familiar with agriculture and does not recognize the indirect benefits of safer and reduced pesticides that the growers can appreciate. This has contributed to the reservations the public has expressed about the use of technology for agricultural products. This is in contrast to pharmaceutical products where the public can take the drug themselves. They have a choice whether to use the product or not. If the product is effective, they experience the direct benefit. In these examples, the public has been much more accepting.

The current situation with nonfood GM products calls for safety and risk assessments that would be accepted by the industry, regulatory agencies, special interest groups, and the public. The risk assessment should be science based and similar to what is used for other products on the market developed by different technologies. A proposed system has been suggested for unintentional exposure. This is based on evaluating risk that is linked to the hazard and exposure (Howard and Donnelly, 2004). Formulas exist for other regulated articles and it has been suggested to modify these equations for nonfood products produced in plants. This would allow a quantitative way to assess the risk of unintentional exposure.

The requirements for producing nonfood products in transgenic plants are considerably different than those used for producing food products. These include physical isolation, delayed planting times from food crops, agronomic support, dedicated equipment, and frequent monitoring. When taking these practices into account, the amount of corn that may inadvertently end up in the food supply and the associated risk can be calculated. In one case, aprotinin, it has been calculated that even without any of the required confinement practices, the amount of aprotinin that could inadvertently end up in the food supply would be a million times below the level needed to show an effect. This means there is no hazard even if the plants were grown and harvested as a commodity crop. If required containment practices are used, these numbers can be drastically lower.

In addition to potential toxicity, some proteins can be allergenic. Using the case of aprotinin, we can calculate how much corn must be eaten to give an antigenic

response. In this example, one would have to eat 350 tacos or 350 bowls of cereal at one time on three different occasions just to get the minimum dose needed to observe an immune response. These calculations seem inconsistent with the fact that the general public considers these non-food products a grave danger.

The example of aprotinin provides one case where even though the product is intended for non-food applications, the product is already in the food supply and no problems have ever been documented. Other proteins discussed earlier—trypsin and avidin—are also already in the food supply and we would anticipate similar results. Clearly, the fear of products getting into the food supply is unwarranted for proteins that are already in the food supply at much higher concentrations. This is not to say that all proteins would have the same risk profile. However, this does point out why a case-by-case assessment is needed and why the public's fears may not be warranted when applying their concerns to all nonfood products.

In conclusion, the technology to produce nonfood products in plants is viable, and the first human health products are now on the market. The advantages of direct delivery have been demonstrated with plant-based oral vaccines. The benefits of this technology are that it may allow many new products to market that are cost effective, convenient, and free of human pathogens. Confinement practices can reduce unintentional exposure, orders of magnitude below the slightest concern for food safety, for many proposed products. However, safety assessment models need to be standardized and accepted by the public, regulatory agencies, and by special interest groups. Finally, we need to treat plant-made pharmaceuticals and plant-made industrial proteins with the same considerations as other pharmaceutical production systems such as eggs or yeast, and not as value-added agriculture.

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