Large-scale Molecular Farming of Recombinant Human Collagen in Transgenic Tobacco

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Collagen and gelatin in medicine, drug delivery, and cosmetics

Through provision of unmatched structural integrity together with cell and substrate anchorage ports, collagen fibers of the extracellular matrix represent macromolecules critical to embryonic development, tissue regeneration, and continual physical support of vertebrates and other multicellular organisms. Type I collagen, the prototype of fibrillar collagens, is the most ubiquitous collagen species in bone and tendon, and is found in significant quantities in skin, aorta, and lung, where variations in fiber diameter, orientation, and packing density dictate the mechanical properties of each tissue. Its primary role in skin structure and support is clearly highlighted upon loss of skin elasticity and strength as a consequence of age-related collagen degradation or crosslinking. Additionally, throughout the multi-stage wound healing process, collagen and collagen-derived fragments provide indispensable support for cell aggregation and adhesion, clot formation, fibroblast recruitment, and adequate scar tissue generation. Biocompatible collagen-based wound dressings or tissue substitutes contribute local haemostatic and chemotactic stimuli, while supplying a structural support upon which neotissue can be formed at enhanced rates. In addition, the highly absorptive character of such products accommodates the high exudate volume characteristic of injured tissue, providing a dry wound bed.

The gelatinous form of collagen, comprised of a mixture of irreversibly denatured collagen chains, features characteristics suitable for applications in food and beverage, pharmaceutical, photographic, cosmetic, paper manufacturing, and printing disciplines. Gelatins are used as binding, microencapsulation, and coating agents for pharmaceutical or health supplement preparations, and owing to their unique breakdown, can also be used to ensure slow release of active ingredients. In addition, their emulsifying effect can easily be manipulated by concentration and temperature. Gelatin sponges and films are marketed as water-insoluble, absorbable medical supplies tailored to control bleeding and offer scaffolding support during early tissue regeneration processes. Expanding appreciation for the advantageous potential of collagen and its byproducts in technologies designed to restore dental, orthopedic, and cosmetic impairments is evident when considering the number of applications being introduced to the market.

Multiple post-translational modifications of collagen: a challenge to recombinant protein expression systems

The type I collagen heterotrimer is composed of two alpha 1 and one alpha 2 chains, constructed from repeating Gly-X-Y triplets, where X and Y can represent any amino acid but are typically proline and hydroxyproline. The polypeptide chains assemble to form a procollagen molecule within the rough endoplasmic reticulum (ER) assisted by the globular C-terminal extension propeptides, forming a trimeric molecule. The complex then folds in a C-to-N direction to yield a triple helix.

Procollagen biosynthesis involves a number of co- and post-translational modifications, including proline and lysine hydroxylation, glycosylation, and disulfide bond formation, all essential for the assembly and physiological stability of the final triple helix conformation. The enzymes responsible for these modifications act in a coordinated fashion to ensure appropriate folding and assembly of a correctly aligned and thermally stable triple-helical molecule.

Stability of collagen’s triple-helical structure in mammals requires prolyl-4-hydroxylase (P4H) activity to form hydroxyproline residues within the collagen chains. Although plants are capable of synthesizing hydroxyproline-containing proteins, plant-derived prolyl hydroxylase exhibits relatively loose substrate sequence specificity in comparison to mammalian P4H. Coexpression of collagen and mammalian-derived prolyl-hydroxylase in insect, yeast and plant cells supports the formation of stable, hydroxylated collagen.

Further posttranslational modifications of collagens involve the lysyl hydroxylase, galactosyltransferase and glucosyltransferase enzymes, which sequentially modify lysyl residues to hydroxlysyl, galactosylhydroxlysyl,
and glucosylgalactosyl hydroxylysyl, respectively. These lysyl carbohydrate structures are unique to collagen and have been implicated in the control of fibril diameter. The human Lysyl hydroxylase 3 (LH3) enzyme can consecutively catalyze all three modification steps required for hydroxylysine-linked carbohydrate formation. In contrast, amino acid analysis of tobacco-expressed human collagen demonstrated hydroxylysine content is less than 2% of that found in bovine collagen, suggesting that endogenic plant lysyl hydroxylase is unable to sufficiently hydroxylate collagen lysines.

Expression of Type I collagen in transgenic systems
Historically, collagen products used for pharmaceutical or biotechnological applications have been extracted from animal or cadaver sources. However, use of such materials can provoke immune responses and involves risk of contamination with pathogens. Alternatively, bacterial and yeast expression systems are inexpensive and appropriate for certain proteins, yet often lack modifying enzymes and molecules required by complex proteins to reach full maturity. The complex biosynthesis of collagen, involving a relatively large number of enzymes managing its expression and maturation, imposes considerable demands on expression systems. As P4H is essential to protein stability, the ideal model must express mammalian variants of the enzyme in compartments that will ensure collagen-enzyme interactions.

The absence of enzymes and co-factors necessary for proline and lysine hydroxylation in traditional microbial expression systems, as well as lack of disulfide bridge formation in microbial cytoplasm, make them unfit for expression of functional collagen.

Using a yeast-based fermentation system, FibroGen Inc. has developed recombinant human collagen type III and gelatins applied toward a gamut of pharmaceutical and medical device applications. In parallel, mammalian cell lines have been proven effective in induction of procollagen expression and secretion, yet require mass culturing volumes, costly nutrient supplementation, and extensive time-to-product periods, while under constant threat of sample contamination by host pathogens. High yields of fully mature collagen are secreted in the milk of transgenic animals bearing mammary gland-targeting genomic collagen- and P4H-encoding inserts. However, the extensive development costs of such models limit significant progress of this expression system. Because protein synthesis pathways are highly conserved between plant and eukaryote systems, the plant can often effectively support expression of complex eukaryotic proteins.

Molecular farming in transgenic plants: a new industry
The recent biotechnology boom has the potential to introduce a wealth of pharmaceutical products and devices to the healthcare market. However, the costly infrastructure and limited production capacity associated with expression of recombinant molecules via microbial fermentation or mammalian cell expression systems hinder realization of the majority of these potential products. The advent of plant-made-pharmaceuticals involving genetic manipulations, programming plants to express molecules of therapeutic value, has introduced a feasible alternative to conventional, fermentation-based expression models. Plant engineering offers cost-effective, safe, manipulable, and easily scalable protein yields harvestable after culture periods significantly shorter than in other expression systems.

While biopharming has sparked much debate over concerns relating to food chain contamination, gene flow, and quality control guidelines regarding fungal toxins and pesticides, tight regulatory supervision has led to significant promotion of this discipline, which is critical to researchers, patients, and farmers. The absence of human and animal pathogens in plants offers an added feature to the use of such systems. The risk-benefit ratios are further enhanced when considering the tobacco plant as a model for production of biologics at commercial-scale levels. As a non-food crop with a large leaf mass and prematurity stage harvesting, both concerns of food supply contamination and of gene flow can be avoided. The decline of traditional tobacco agriculture followed by the search for novel farming opportunities has prompted the harnessing of tobacco plant production capacity toward meeting the growing demand for biologics.

Both plant nuclei and chloroplasts are exploited for high-yield expression of proteins, biopharmaceuticals,
antibodies, and vaccines that demonstrate structural and posttranslational specifications closely resembling those of their natural counterparts. However, lack of specific enzymatic support often leads to plant-derived recombinant molecules void of modifications critical to their half-life and activity. Subcellular targeting of recombinant protein expression represents an additional factor influencing the maturity and yield of biologically relevant products expressed in the plant. Collplant Ltd. reported the integration of a matrix of distinct plant features toward development of a high output system for expression of hydroxylated, heterotrimeric, recombinant human procollagen type I (rhPCOL1). Through a series of crossbreedings, a tobacco line was engineered to coexpress vacuole-targeted human procollagen alpha 1 and alpha 2 chains together with human posttranslational modifying enzymes P4H alpha, P4H beta, and LH3. The final product, expressed in a compartment free of homologous plant enzymes or potential P4H inhibitors, proved viable and active. The purified and processed heterotrimeric recombinant human collagen (Collage rh™) product is thermally stable, demonstrates fibril-forming capacities, and supports the attachment and expansion of various primary human cells normally involved in tissue repair processes (Fig. 1).

Figure 1. Enhanced cell proliferation on Collage rh™ scaffolds
Primary human keratinocytes, endothelial cells and dermal fibroblasts (25 x 10^3 cells/well) were seeded on 6 mm diameter scaffolds that were placed in 96-well tissue culture plates. The scaffolds used were composed of bovine collagen, bovine alginate, bovine cellulose or recombinant human collagen (Collage rh™). Negative control samples included cell media only or scaffolds in media, with no cells. Cells were allowed to proliferate on scaffolds for 72 h (37°C, 5% CO₂). Scaffolds were then transferred to clean wells, supplemented with fresh media and WST-1 proliferation/viability reagent (1 hr, 37°C, 5% CO₂). Absorbance, indicative of viable cells, was determined (450 nm) using ELISA plate reader. Means of triplicate samples are presented (+/-SD). * Student's t-test: p<0.05. (Source: Large-scale molecular farming of recombinant human collagen in transgenic tobacco, Oded Shoseyov et al.)
Conclusions
Novel biopharming ventures feature a multitude of revolutionary prospects. Increased awareness of the plant-based protein expression system, farmer education, and appropriate regulatory policies and measures will boost full exploitation of these highly manipulable and cost-effective natural bioreactors. More specifically, affirmative actions taken to facilitate such agricultural and technological opportunities can reverse the unfavorable public image of tobacco. Such measures will stimulate exploitation of the tremendous protein production advantages innate to the tobacco plant and will reestablish its credibility as a legitimate crop with the potential of benefiting mankind.

References

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