Potential of a Bio-Coll@gen™ in Dermatology and in Cosmetics

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Abstract

Background

Collagen has been widely used as a major component for dermatology and cosmetic formulations because of its significant benefits as a natural ingredient and moisturizer. Animal-derived collagen suffers drawbacks such as risks of infectious disease and virial transmissions, allergic reaction, and unpleasant smell/color; however it is relatively abundant and affordable. Thus, the customers are constantly looking for innovative, more sustainable, and truly efficacious products.

Methods

In this study, we report the production of a 50 kDa protein in a eukaryotic host that is 100% identical to human type III collagen sequence. This recombinant protein has been evaluated in vitro in biostimulation of Collagen III. Safety profile and Clinical studies have been performed to evaluate its efficacy.

Results

We report the successful industrialization of both the fermentation and purification processes for production of a final recombinant protein product. This protein was shown to be safe for application to human skin. It was also shown to be compatible with all common formulations.

The recombinant 50 kDa collagen type III protein was shown to uniquely stimulate collagen type III production and secretion by primary human dermal fibroblasts. Clinical studies have shown a benefice in anti-ageing with increase of the collagen content, barrier function and skin density. Clinical experts have scored an improvement of different parameters including firmness, lines and wrinkles.

Conclusion

The unique combination biostimulation, clinical studies and demonstrated commercial production make this novel recombinant Type III collagen a good candidate for broad application usage in dermatology and in the cosmetics industry.

Keywords

collagen III; Pichia; anti-ageing; dermatology; cosmetics; fermentation

1.Introduction

Collagen is the most abundant protein in the human body, and present in connective tissue such as cartilage, bones, tendons, ligaments, and skin [1]. The collagen superfamily comprises twenty-eight members in which type I collagen is the most abundant form (~80-85% of total collagen) and major protein in the extra-cellular matrix of human cells. It assembles into fibers that form the structural and mechanical scaffold (matrix) of skin and other connective tissues.

Collagen type III is the second abundant collagen (~10-15% of total collagen) that is primarily produced by young fibroblasts before the tougher type I collagen is synthesized [2]. Collagen III is found in granulation tissue and artery walls, skin, intestines, uterus as well [3, 4, 5]. Both collagen I and III are fibrillar collagens that form higher order structures (fibril, fiber and bundle) but they have slightly different structural properties. Type I collagen tends to form densely packed fibrils and a rigid triple helix for structure building. On the other hand, type III collagen forms a more flexible triple helical structure that assembles into small fibrils that are associated with the more rigid collagen I fibrils. As a result, collagen III functions as a "modulator" of overall tissue elasticity and function. Indeed, the ratio of collagen I/III correlates with skin aging and elasticity [6, 7, 8, 9].

Type I collagen is frequently used as a supplement in cosmetics products. While most commercial forms of collagen come from mammals, such as bovine or porcine, other animal sources such chicken, fish skin, and jelly fish have also been reported [10, 11, 12, 13, 14, 15, 16]. Although animal-derived collagen is generally abundant and cheap it does suffer drawbacks such as risk of infectious disease transmission, potential viral vector transmission from animal to human, allergic reaction to human body and unpleasant smell and color [17]. Non-type I

collagens, such as Type III collagen tend to be very rare and expensive due to challenges with sourcing sufficient protein quantities and purifying from natural sources [18].

In the last two decades, with the advent of and advancement of genetic and cell engineering, progressively more effort has been put into recombinant collagen production. Recombinant collagen molecules of different sizes have been expressed in all major expression platforms including mammalian cells, insect cells, yeast, bacteria and plant [19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37]. In general, high quality full-length collagen proteins have been produced in eukaryotic hosts but with very low productivity. Prokaryotic hosts, such as *E.coli* were also explored for production of unmodified collagen, however these recombinant proteins were generally either very small (a few kDa up to 20 kDa) or have been extensively modified from wild-type human collagen sequences. It is well known that prokaryotic hosts lack post translational modification functions that are needed to generate the hydroxyproline amino acid residues that are found in mature animal derived collagen. As such, previously produced recombinant collagen proteins generally only exhibit an unmodified collagen sequence [38, 39, 40].

In this study, we report the production of a 50 kDa protein in a eukaryotic host (*Pichia Pastoris*) that is 100% identical to human type III collagen. We successfully scaled the production to industrial scale and the final protein prep has been shown to be safe to human skin and it is neither allergenic nor mutagenic. It is also compatible with all formulations protocols we have tested. Unexpectedly, this protein stimulated collagen III synthesis in primary human dermal fibroblast assays. Clinical studies have been performed to evaluate the efficacy of this exciting new bioactive.

2. **Materials and methods**

2.1.Formulation Compatibility Evaluation

The testing materials were each dissolved at a 1% by weight concentration in Deionized Water, The resulting solution were added at 3% by weight into seven base formulations to test compatibility and performance: Gel (Carbomer System); Serum (Ammonium Acryloyldimethyltaurate/VP Copolymer and Acrylates/C10-30; Alkyl Acrylate Crosspolymer System); Cream Emulsion (Oil/Water); Hair Conditioner (Containing Cetrimonium Chloride and Behentrimonium Methosulfate); Shampoo/Cleanser (Sulfate-Free); Toner (Water-based System); Toner (Alcohol-based System).

2.2. Safety Testing

EpiOcular

EpiOcular Irritation Test was performed using previously used protocol [41].

HRIPT

The human repeated insult patch test (HRIPT) was performed using the standard protocol [42].

Bacterial Reverse Mutation Assay

Bacterial Reverse Mutation Assay (Ames Test) was done according to previously used protocol [43].

2.3. Efficacy in vitro

MTT assay

Primary human dermal fibroblast cells were seeded in a petri dish. After a 2-day incubation, the cell culture medium was removed, and the fibroblasts were washed twice with PBS to remove

any remaining test material. After the final wash, 500 µl of DMEM supplemented with 0.5 mg/ml MTT was added to each well and the cells were incubated for 1 hour at 37°C and 5% CO_2. After the incubation, the DMEM/MTT solution was removed, and the cells were washed again once with PBS and then 0.5 ml of isopropyl alcohol was added to the well to extract the purple formazin crystals. Two hundred microliters of the isopropyl extracts were transferred to a 96-well plate and the plate was read at 540 nm using isopropyl alcohol as a blank. The mean MTT absorbance value for the negative control cells was calculated and used to represent 100% cell viability. The individual MTT values from the cells undergoing the various treatments were then divided by the mean value for the negative control cells and expressed as a percent to determine the change in cell viability caused by each treatment.

Type III Collagen Assay

A series of standards were prepared and 100 ul of these standards or samples were added to the wells of the type III collagen ELISA plates. The plates were then incubated at 37°C for 1.5 hours. After this incubation the ELISA plates were then washed twice with wash buffer, followed by the application of 100 ul of detection antibody solution. The ELISA plates were then incubated for 1 hour at 37°C. After incubation all of the ELISA plates were washed with wash buffer followed by the addition of 100 ul of HRP conjugate solution and incubated at 37°C for 30 minutes. After this incubation the ELISA plates were again washed and 100 ul of substrate solution was added to each well and the well-plates were incubated for 10-30 minutes at room temperature to allow the color generation reaction to occur. At the end of the color generation reaction 100 ul of stop solution was added to each well and the plates were read at 460 nm using a plate reader.

2.4. Efficacy in vivo

Clinical studies have been performed to evaluate the efficacy of the Bio-Coll@gen™ in anti-ageing with instrumental quantification of the collagen content with the device SIAScope, the barrier function with the Vapometer and the skin density with device Derma Lab Ultrasound. In addition, clinical experts have scored the improvement of different parameters with visual assessments through photos with the device Colorface using a 10-point scale. The satisfaction of the consumers has been investigated with a questionnaire, following the "standard guide for sensory claim substantiation" [44].

3.Results

3.1. Production of 50 kDa protein

The production of recombinant 50 kDa protein is summarized in Figure 1. We first generated a plasmid containing a strong promoter, a secretion signal, and a portion of a codon optimized human Col3A1 gene. The circular plasmid was linearized and transformed into Pichia Pastoris cells and transformants were selected on YPD plates with antibiotics.

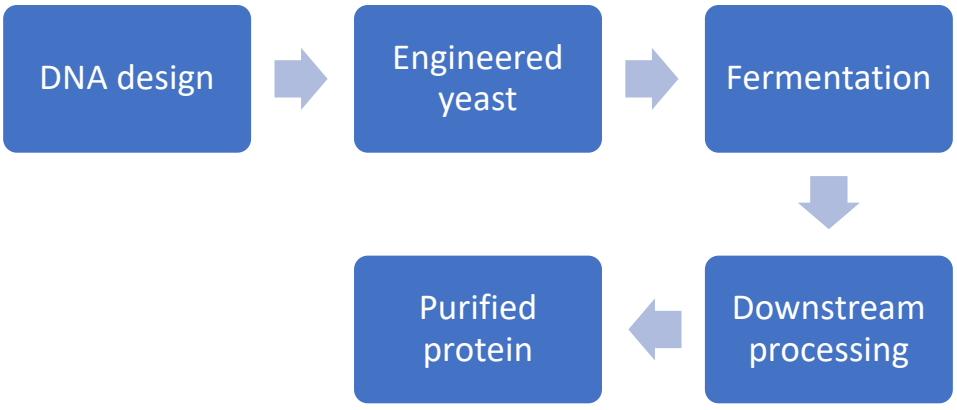

Figure 1. Workflow to produce 50 kDa protein

The best clone was obtained by several rounds of screening and then promoted into fermentation development. A three-step fermentation process was started by inoculating the best clone in shake flasks, followed by a seed fermentation lasting approximately 23-27 hours, which was then used to inoculate a production fermenter. The production fermenter was run for about 72 hours with a 24-hour batch phase followed by a 48-hour fed-batch phase. At the end of fermentation, yeast cells were removed by centrifugation and the supernatant containing target protein was processed to further purify the recombinant protein.

3.2. Formulation testing and potential applications

We tested various formulations to determine its properties and potential in various personal care applications and to assess its market potential as compared to currently available commercial collagens. Evaluation included technical analysis of physical properties, compatibility with various formula chassis types, sensorial characteristics, stability, and finally solubility, to create a broad understanding of potential applications.

Based on the sensorial and solubility findings, we believe the recombinant 50kDa Type III protein would be viable in a broad array of potential applications in using common personal care formulations. Notably, the bioactive protein exhibited good color and odor and could easily be incorporated into certain formulas. As such, we believe this material could be potentially formulated into the major categories: skin care, body care and color cosmetics.

3.3. Safety testing

Ocular irritation and HRIPT were completed to verify the expected safety performance of the 50kDa protein. Ocular irritation is defined as reversible damage to the eye following application

of a test substance, whereas ocular corrosion is defined as irreversible damage. Irritation and sensitization (contact allergy) potential was evaluated via the typical HRIPT, which entails repeated application to the skin of human subjects. We also assess the risk of the protein to induce bacterial reverse mutation using the classical Ames test. This test was used to evaluate the mutagenic potential of the test sample at a 1% concentration of the test sample. The HRIPT (Human Repeat Insult Patch Test) study has shown that under the conditions of the study, there was no indication of a potential to elicit dermal irritation or sensitization (contact allergy) noted for this protein prep. The data from EpiOcular test predicted that this protein to be classified as a GHS NC, with an average viability score of 85.3% and passed quality control checks. In the Ames mutagenic potential evaluation, this protein was found to be not cytotoxic to the test system and no detectable genotoxic activity associated with this protein at 1%.

3.4. Collagen synthesis induction by 50 kDa protein

We used a fibroblast cell culture model to assess the ability of the recombinant 50 kDa protein to exert an effect on collagen synthesis. Fibroblasts are the main source of the extracellular matrix peptides, including the structural proteins collagen and elastin.

The viability of the cells after exposure to the protein prep was first assessed by the MTT assay. The MTT assay is a colorimetric analysis of the metabolic activity of the cell, which is a reflection of the number of viable cells.

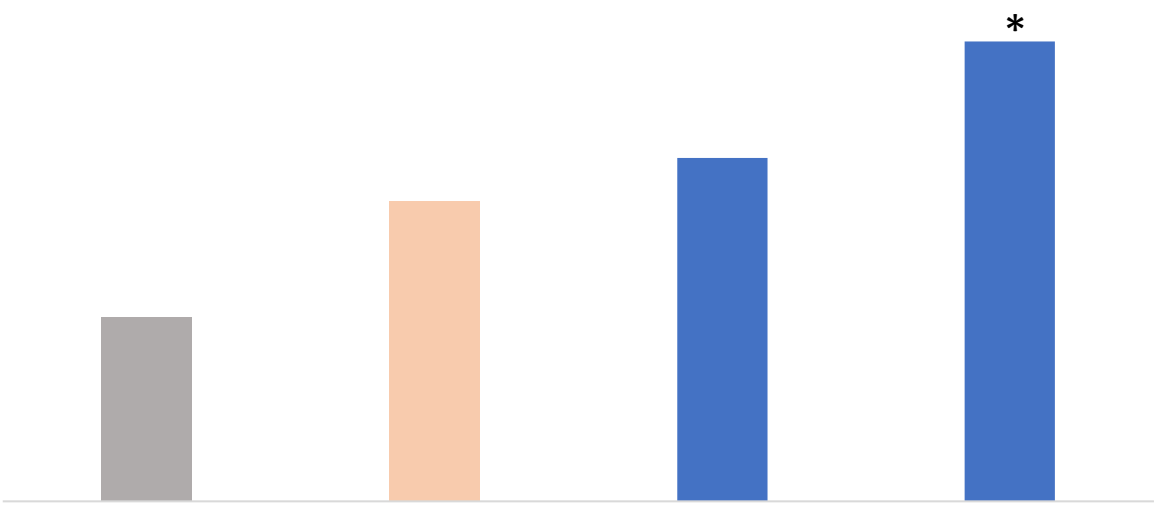

Figure 2. Collagen type III assay

The recombinant 50 kDa protein stimulated collagen III production in fibroblast. The protein prep at both 0.1% and 0.05% significantly increased soluble collagen III synthesis more effectively than TGFβ1 control (Figure 2). The commercial marine collagen samples had no or some negative effect on collagen III synthesis at the same concentrations.

3.5. Evaluation in vivo .

Clinical studies have demonstrated the efficacy of the Bio-Coll@gen™ in anti-ageing. Instrumental quantifications have proven an improvement of the collagen content, of the barrier function and the skin density (figure 3)

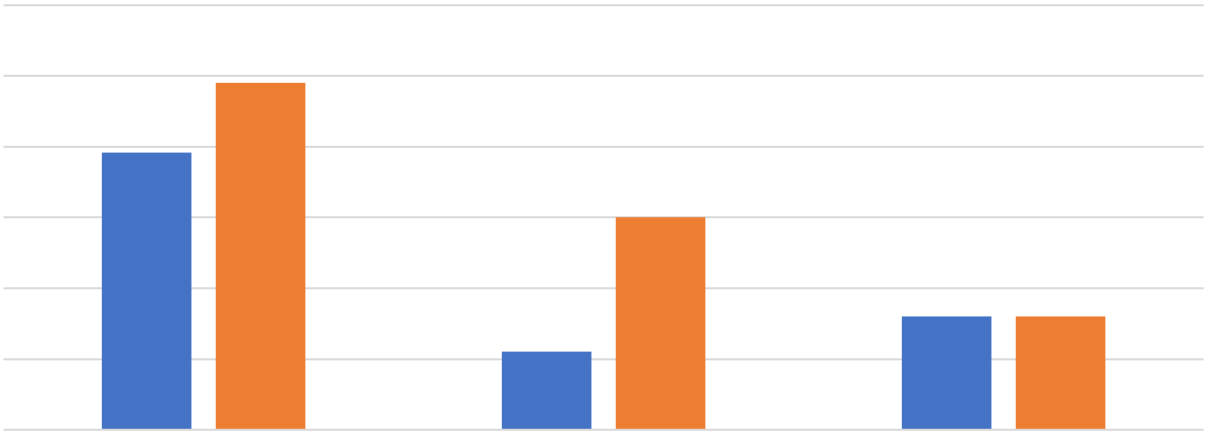

Figure 3: Instrumental Evaluation

In addition, clinical experts have scored the improvement of skin firmness and elasticity (25%), appearance of lines and wrinkles, (18%) smooth skin texture (18%) and appearance of sagging skin (27%).

The satisfaction of the consumers has been measured over 75% for the majority of the questionnaire.

4. Discussion

This study illustrated the production of a 50 kDa recombinant protein from a eukaryotic host. Unlike many other recombinant collagen-like proteins, this protein is 100% identical to human collagen type III alpha chain without any sequence manipulation and will mimic natural collagen III function at the highest level. Given the close correlation between aging and collagen I/III ratio, this protein could well balance the collagen composition and thus elasticity in aged skin.

Because this protein is produced in single-cell organism (yeast) we are able to easily scale the fermentation to industrial scale to reduce cost. We used simple fermentation media and predominantly physical separation to purify the final sample, the resulting protein preparation is highly pure and free of harmful chemicals (strong acid or base), which are often seen in animal-derived collagen preparations.

Many collagen samples suffer from color, odor, solubility and ability to formulate as a potential personal care product. Our 50 kDa final protein is odorless and light in color at high concentrations. When the protein was tested in various formulation protocols, it was compatible with all formulations including ethanol-based protocols. Furthermore, from a safety perspective the Ocular Irritation, HRIPT and Ames tests results verified the safety of the protein for personal care applications.

This 50 kDa human collagen protein was able to enhance collagen III production in fibroblast comparable to or even better than the TGFβ1 positive control. [45].

Clinical studies have been shown the anti-ageing benefits of Bio-Coll@gen™ supported by instrumental quantification of the collagen content, barrier function and skin density. In addition, clinical experts have scored the improvement of different parameters and the satisfaction of the consumers has been demonstrated.

All of these properties make this 50 kDa protein a good candidate for personal care product development. There are many known uses for collagen in the cosmetics and skincare industry, for example, skincare compositions that include collagen can be used to combat the effects of aging and environmental stress on the appearance, elasticity, and thickness of skin [46]. For example, ageing and environmental factors can lead to dermatological conditions including, but

not limited to fine lines, wrinkles, dry skin, excessive pore size, skin dyschromia, reduced elasticity, unwanted hair, skin thinning, purpura, actinic keratosis, pruritus, eczema, acne, rosacea, erythema, telangiectasia, actinic telangiectasia, skin cancer, and rhinophyma. Although there are numerous skincare products on the market to improve skin appearance, many consumers are hesitant to use chemically synthesized products they perceive as being environmentally unfriendly or otherwise unsafe. This protein also provides a potential route for treating a wide range of conditions. Oral collagen supplements and drinks have been shown to improve skin hydration and elasticity for older people [48, 49, 50]. Since this protein was produced in a natural yeast host and purified mostly by physical separation, it is a safer and more environmentally friendly substitute for animal-derived collagen. To achieve these goals, this recombinant protein can be formulated into various formulations.

5. Conclusion

We reported the production of a recombinant 50 kDa protein in yeast at large scale. This protein is safe to human skin, compatible with many different formulations, and stimulates collagen III synthesis in human dermal fibroblast. Clinical study results have supported the benefits of the Bio-Coll@gen™ in anti-ageing. The 50 kD protein and its positive outcomes has the potential for broad cosmetic applications.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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