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Recombinant human collagen III from moss protonema cultures: A new vegan collagen active

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1. Introduction

Human collagen is the predominant structural protein of the extracellular matrix and a key component of connective tissue, where it contributes to strength and elasticity [1,2]. In human skin, collagen is of fundamental importance, giving smoothness and elasticity, though its production declines with age [3]. Collagen loss is in fact not only a consequence of aging, but also one of its causes [4]. Therefore, it is important to replenish and protect collagen to counteract skin aging. Traditionally sourced from animals, collagen is widely used, especially as an ingredient in dietary supplements and cosmetic products and faces growing demand. However, safety concerns, along with increasing consumer interest in ethically sourced and environmentally friendly ingredients, have driven a shift toward animal-free alternatives containing recombinantly produced collagen [5]. Other than collagen I, which is a stiff protein giving tensile strength to the skin, collagen III is crucial for providing the skin with elasticity. It is especially important in wound healing, as it forms a temporary scaffold that supports the migration and proliferation of cells necessary for tissue repair [6]. These thin fibers have a high turnover rate and influence the maturation of the thicker type I collagen [7]. Moreover, by interacting with other molecules in the extracellular matrix, such as elastin and hyaluronic acid, it ensures optimal skin structure and moisture retention [8]. To meet the demand for vegan, halal, and environmentally friendly alternatives to animal-derived collagen in cosmetic applications, we

explored the potential of the moss Physcomitrella (*Physcomitrium patens*), a well-established model organism in plant biology and biotechnology [9], for the production of a recombinant human collagen type III peptide. With a proven track record as a production platform for biopharmaceuticals [10], Physcomitrella offers several key advantages. It can be cultivated in bioreactors under Good Manufacturing Practice (GMP) conditions [11]. Moreover, its ability to secrete recombinant proteins directly into a simple culture medium significantly reduces the complexity of downstream processing by eliminating the need for tissue extraction and extensive purification [12,13]. Physcomitrella is also highly accessible to precise genome engineering [14] and naturally performs prolyl 4-hydroxylation through six distinct P4H enzymes [15]. In the context of collagen, prolyl 4-hydroxylation is a critical post-translational modification that ensures structural integrity, thermal stability, biological functionality [16]. Further, it plays an essential role in maintaining tissue homeostasis [16] and is considered a key quality marker for recombinant collagen. Unlike microbial systems such as *Escherichia coli* (*E. coli*) and yeasts, which entirely lack the machinery for prolyl hydroxylation [17,18] or plant-based production platforms such as tobacco [19], corn [20], and barley [21] that contain insufficiently specific endogenous P4H enzymes [22], Physcomitrella provides with its six P4H enzymes a significant advantage for achieving this crucial modification without further metabolic engineering. In addition to targeting a high biomimetic quality, specifically characterized by the presence of prolyl 4-hydroxylation, we also focused on achieving favorable solubility properties to facilitate the development of a secretion-based production process and enhancing the peptide´s suitability for a wide range of cosmetic formulations. The resulting collagen peptide-rich culture supernatant demonstrated efficacy in modulating gene expression related to collagen synthesis, extracellular matrix modulation, and skin regeneration in both epidermal keratinocytes and dermal fibroblasts, as well as in stimulating hyaluronic acid release. Taken together, this study highlights the potential of Physcomitrella as a sustainable production platform that meets the growing demand for animal-free, ethically sourced high-quality collagen peptides in cosmetic applications and introduces a novel collagen-based active ingredient to target signs of skin aging.

2. Materials and Methods

2.1. Production and quality assessment of a recombinant collagen peptide in the moss Physcomitrella

To provide an animal-free collagen alternative, a 334-amino-acid hydrophilic region of human type III collagen was selected and its DNA sequence optimized for expression in Physcomitrella. The corresponding encoding DNA sequence (CDS) was *in silico* optimized for expression in Physcomitrella and fused to the CDS of a signal peptide to enable product secretion as well as to a His-tag encoding sequence for potential purification purposes. Stable lines were

generated via polyethylene glycol (PEG)-mediated protoplast transfection and screened using Western blot to detect recombinant collagen in tissue extracts or culture supernatants. Proteins were isolated using His SpinTrap columns (Cytiva) according to the manufacturer's instructions or culture supernatant samples were precipitated using acetone. The respective proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed with an anti-His primary antibody (1:2,000), followed by an HRP-conjugated anti-mouse secondary antibody (1:10,000). Confirmation of collagen peptide identity was additionally performed via immuno-detection with an anti-collagen primary antibody (1:1,000) and a subsequent detection with an HRP-linked anti-rabbit secondary antibody (1:5,000). The quality of the intracellular as well as secreted collagen peptide was evaluated using mass spectrometric analysis. Subsequently, a secretion-based production process was developed and scaled up to 5-liter photobioreactors. Quantification of the extracellular collagen peptide accumulated in the culture supernatant, which represents the final product intended for use in cosmetic formulations, was performed using an established anti-His antibody-based ELISA (Enzyme-Linked Immunosorbent Assay). In brief, supernatant and standard samples were diluted and used to coat the wells of a micro-titer plate. After blocking, wells were incubated with an anti-His primary antibody (1:2,000), followed by a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:2,000). Colorimetric detection was then performed to quantify the collagen peptide concen-tration.

2.2. Efficacy testing

2.2.1. Gene expression analysis

The cells, either Normal Human Epidermal Keratinocytes (NHEK) or Normal Human Dermal Fibroblasts (NHDF), were cultured in appropriate cell culture medium at 37 °C and 5 % CO_2. The samples were diluted in cell culture medium, and cells were treated or not with the Physcomitrella culture medium, containing the collagen peptide, for 24 hours, working in tripli-cates for each condition. Afterwards, cells were harvested for mRNA extraction. mRNA was extracted and reverse transcribed into cDNA. After a pre-amplification step, each sample was amplified using real-time PCR on a special chip for the BioMark® PCR system (n=2). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, normalizing each gene by the reference genes and comparing the expression to the untreated control. For each condition, the standard error of mean (SEM) was calculated and data with SEM > 40 % (mean of all 6 values) was removed from the analysis.

2.2.2. Hyaluronic acid release

For this assay, normal human foreskin-derived dermal fibroblasts were cultured in cell culture medium in a humified incubator at 37 °C and 5 % CO_2. To assess the release of hyaluronic

acid, an ELISA was performed according to the manufacturer's protocol. In brief, standards and samples were pipetted into the wells of the ELISA plate to bind any present hyaluronic acid. After washing, an antibody specific to human hyaluronic acid was added, followed by a substrate solution to quantify the amount of proteins bound in the initial step. The absorbance was measured by colorimetry and the quantification of hyaluronic acid release was performed based on a standard curve. The effects of the treatments were compared to untreated cells as control and to the reference Oleoyl-L-α-lysophosphatidic acid (LPA) sodium salt at 6 µM (Sigma Aldrich, USA). The data was normalized to cell viability, which was measured through a preliminary cytotoxicity test (MTS assay).

3. Results

3.1 Production of a 30 kDa prolyl-hydroxylated recombinant collagen peptide by transgenic Physcomitrella lines

In the initial screening step, transgenic Physcomitrella lines were assessed for recombinant collagen peptide production. Total protein was extracted and analyzed using anti-His tag antibody-based immunodetection in which a specific signal at around 45 kDa which was present in the transgenic lines and absent in the wild-type control, confirmed recombinant collagen peptide production in the selected lines C6.1, C5, C11, C13, C15, C34, C44 and C46 (Figure 1 A). To confirm that the 45 kDa signal originated from the recombinant collagen peptide, total protein from line C6.1 was reanalyzed using an anti-collagen antibody, yielding results similar to those with the anti-His antibody (Figure 1B) and verifying the signal's identity. To analyze the quality of the recombinant product, the SDS-PAGE gel band corresponding to the 45 kDa signal was mass spectrometrically investigated, which confirmed the production of the full-length and intact collagen peptide (Figure 1 C). Additionally, the Physcomitrella-derived collagen peptide was found to be efficiently prolyl hydroxylated (Figure 1 C).

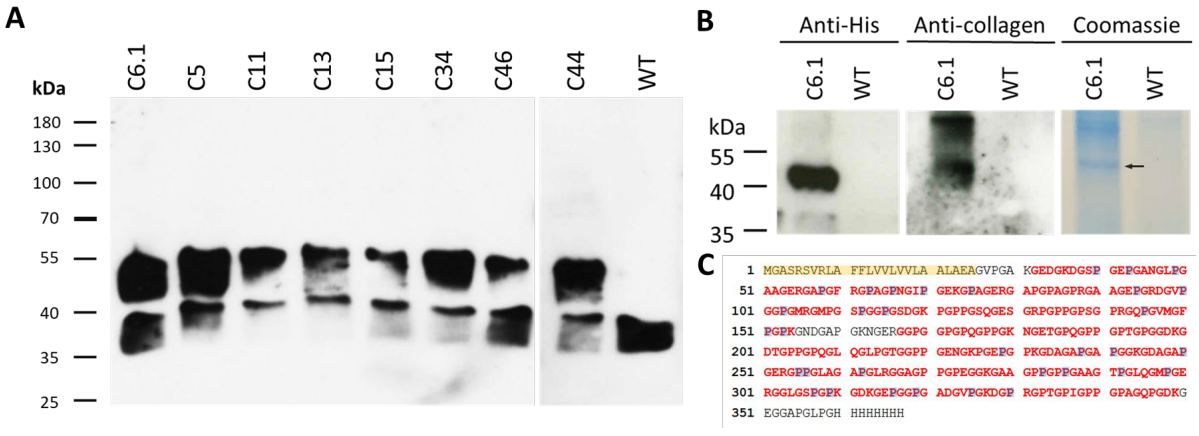

Figure 1: *Analysis of the Physcomitrella-produced recombinant collagen peptide.*

A) Immunodetection of His SpinTrap-purified total protein extracts from protonema tissue of transgenic Physcomitrella lines producing recombinant collagen peptide, compared to a wild-type (WT) control. B) Confirmation of co-localizing signals detected by anti-His and anti-collagen immunodetection of total protein extracts from line C6.1. C) Mass spectrometric analysis of the recombinantly produced collagen peptide. Mass spectrometry of the proteins excised from the SDS-PAGE gel band corresponding to the immunodetection signals revealed 89% sequence coverage of the mature collagen peptide (amino acids shown in bold red) and confirmed the cleavage of the secretion mediating signal peptide (highlighted in yellow). Prolines highlighted in blue were observed in their hydroxylated form.

3.2 Development of a larger scale production

Based on the intended water solubility of the recombinant collagen peptide and Physcomitrella's ability to grow in sterile, additive-free medium and release proteins into the supernatant., a secretion-based process was developed . In the initial establishment phase, key parameters, such as medium composition, pH, CO_2 supplementation, and biomass density, were systematically optimized and then applied to 5-liter photobioreactors, yielding over 1000 µg collagen peptide/L in 22 days. By implementing a biomass-dependent and light intensity-regulated secretion process, the production time was halved, resulting in a robust and resource-efficient production process (Figure 2A). The quality of the secreted collagen peptide was assessed via anti-His immunodetection and mass spectrometry, confirming the presence of intact monomers and multimers in the supernatant (Figure 2B). For product recovery, the collagen-containing supernatant is separated from the moss tissue via sequential filtration steps and can be stored long-term at -20 °C prior to formulation. Both in-process and final collagen peptide concentrations were quantified using ELISA.

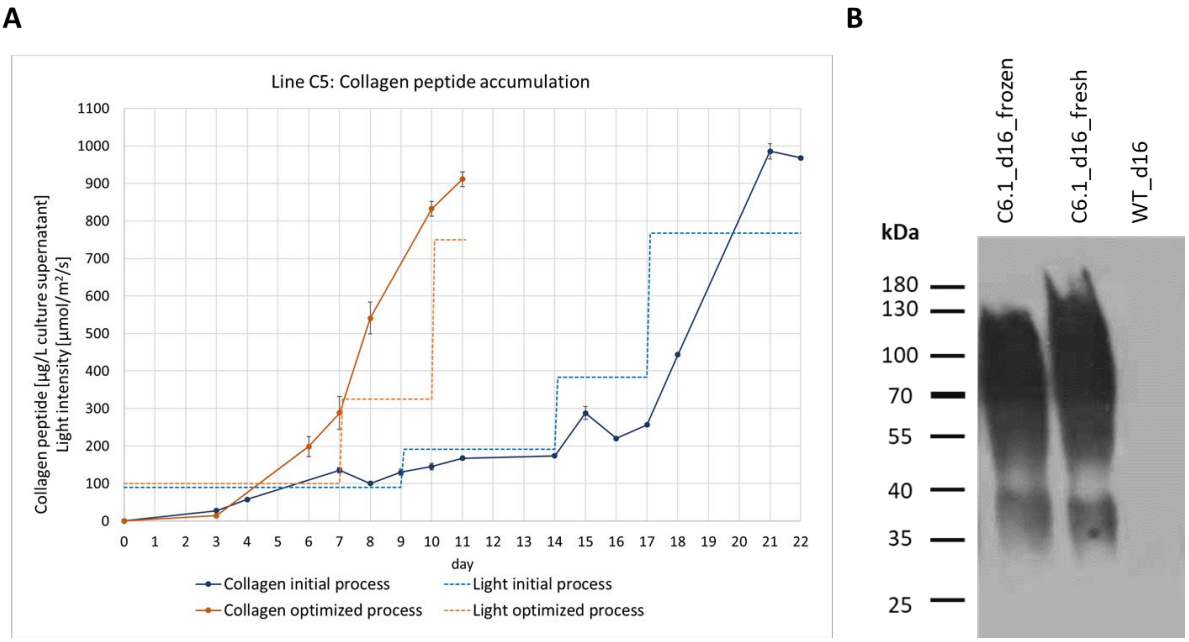

Figure 2: *Culture supernatant accumulation of the recombinant collagen peptide and analysis of the secreted product*.

A) Collagen peptide accumulation profiles (solid lines) and corresponding light intensities (dashed lines) during 5 L photobioreactor runs with the line C5, shown for both the initial and optimized conditions. The optimized process includes a biomass-dependent light intensity profile. ELISA was used for quantification; data represent means ± SD of technical triplicates. B) Anti-His immunodetection of acetone precipitated bioreactor supernatants (1.5 ml) from a 16-day bioreactor run of line C6.1 showed strong monomer signals (~35 kDa) and higher molecular weight bands (45–130 kDa), indicating multimerization. Samples included acetone precipitations from freshly processed as well as from previously frozen supernatant samples, with no signal in a freshly prepared wild-type control of a 16 day long bioreactor run. Mass spectrometry confirmed the presence of recombinant collagen in all detected signal areas.

3.3 Recombinant collagen peptide-containing Physcomitrella culture supernatant enhances expression of anti-aging genes.

To broadly assess the effect on the recombinant human collagen III on skin cells, gene expression of several genes related to optimal skin function was investigated in two different skin cell types. Treatment of NHEK and NHDF with recombinant collagen peptide-containing Physcomitrella culture supernatant led to increased expression of genes important for skin health. On fibroblasts, Physcomitrella culture supernatant enhanced the expression of collagen-encoding genes, while reducing the expression of the pro-inflammatory cytokine IL6. On keratinocytes as well, several collagen-encoding genes were observed to be upregulated, as well as genes encoding for sirtuins and peptide methionine sulfoxide reductases, which repair oxidative damage to proteins.

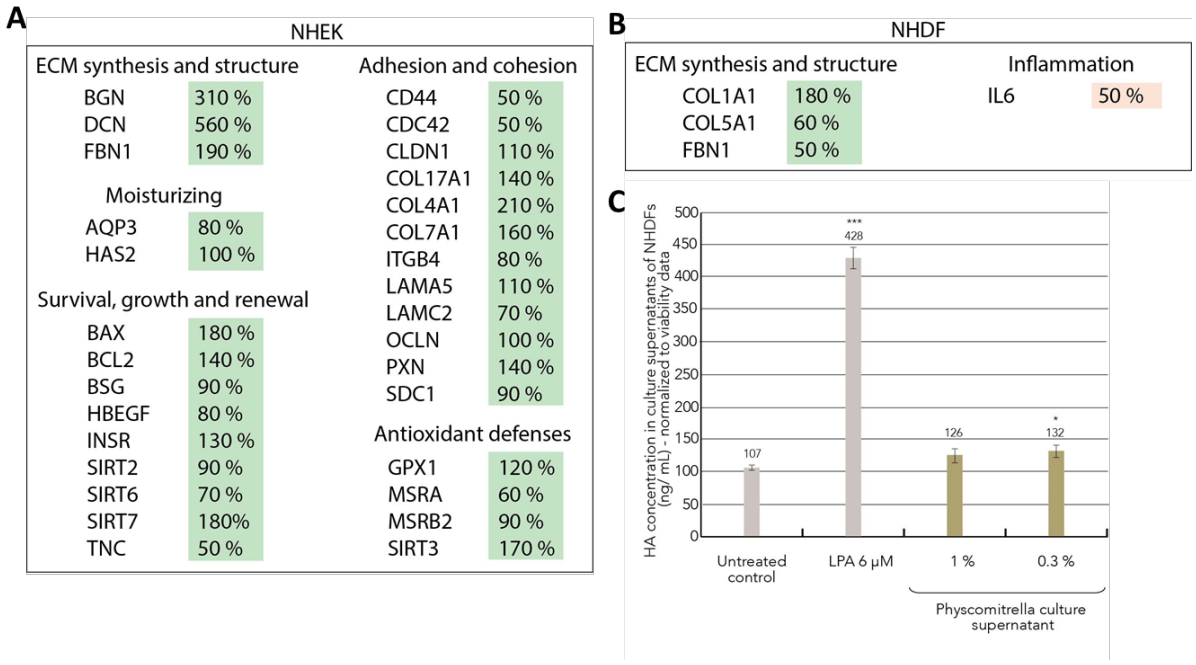

Figure 3: *Impact of the recombinant collagen peptide-containing Physcomitrella culture*

*supernatant on NHDF and NHEK cells. Modulation of gene expression by recombinant collagen peptide-containing Physcomitrella culture supernatant on NHDF (A) and NHEK (B) cells. C) Promotion of hyaluronic acid release of NHDF cells upon the application off different concentrations of the collagen peptide-containing Physcomitrella culture supernatant. *p<0.05, ***p<0.001*

3.4 Recombinant collagen peptide-containing Physcomitrella culture supernatant promotes hyaluronic acid release

To further investigate the effect of recombinant collagen on skin cells at the protein level, hyaluronic acid release by fibroblasts was assessed. On NHDF, the release of hyaluronic acid was increased by 25 % and 19 % upon treatment with 0.3 % or 1 % collagen peptide containing culture supernatant.

4. Discussion

Recombinant collagens are not only gaining popularity in the cosmetic industry as a valuable alternative to animal-derived collagens, offering benefits such as high purity and low immunogenicity, but also align with the growing consumer demand for vegan, sustainable, and ethically sourced cosmetic ingredients [5]. In this study, we describe the synthesis and purification of a 30 kDa prolyl-hydroxylated recombinant human collagen type III peptide in transgenic Physcomitrella lines and demonstrate its efficacy in modulating specific gene expression pathways in epidermal keratinocytes and dermal fibroblasts, as well as in stimulating hyaluronic acid secretion. Using immunodetection and mass spectrometry, we confirmed the presence of full-length, prolyl-hydroxylated collagen peptide in Physcomitrella. Prolyl hydroxylation is a key post-translational modification in collagen, essential for structural integrity, thermal stability, and biological functionality [23,24]. Its presence is therefore considered a hallmark of the biomimetic quality of recombinantly produced collagen derivatives and a major quality goal, which could be addressed with the Physcomitrella-based production system. Unique, compared to other established non-mammalian biotechnological production hosts such as *E. coli* [18,25], yeast [17,26,27], insect cells [28,29] and other plant bases systems [19-21,30] is, that Physcomitrella with its six endogenous P4H enzymes [15] could facilitate efficient prolyl hydroxylation of the recombinantly produced collagen peptide without the need for metabolic engineering. Additionally, the engineered solubility of the collagen peptide, achieved by selecting a sequence with naturally occurring hydrophilic stretches, enabled the development of a secretion-based production process. This process was successfully scaled up to 5-liter photobioreactors, yielding collagen peptide-rich culture supernatants suitable for direct use in cosmetic formulations. Functional assays performed with these supernatants demonstrated their ability to modulate

gene expression in epidermal keratinocytes and dermal fibroblasts, mainly by upregulating the expression of genes involved in skin moisturization, ECM synthesis and structure, antioxidant defenses, cell survival and growth and skin renewal.

Interestingly, Physcomitrella-derived collagen peptide containing supernatant led to the upregulation of ECM components other than collagen III, such as Fibrillin-1, biglycan, dystroglycan and other collagens. This response to collagen-peptide treatments has already been observed in response to other collagen-peptide treatments [31], and might reflect the need to maintain a balance between different ECM components. We also show that Physcomitrella-derived collagen peptide containing supernatant upregulates the secretion of hyaluronic acid. This extracellular matrix component interacts with collagen III to ensure optimal skin structure and moisture retention. It is therefore possible that in this case as well, an optimal ratio between the different ECM components needs to be maintained. It would certainly be worth investigating how the ECM structure and its quality are affected by the treatment with Physcomitrella-derived collagen peptide in a 3D skin model. This could be done for example through histochemical staining of ex vivo samples or on reconstructed human epidermis. Furthermore, clinical testing of the recombinant Physcomitrella-derived collagen peptide on volunteers with aged skin could provide evidence of the peptide's effectiveness in reducing wrinkles and other signs of aging as well as promoting skin function in general.

5. Conclusion

The Physcomitrella-derived recombinant, post-translationally modified human collagen type III peptide provides a green, vegan, sustainable ethically sourced and consumer-friendly alternative to animal-derived sources and elicits an anti-aging effect on skin cells.

6. References

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