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# New Thrombospondin-1-Deriving Peptides as TGF-β Activators of Cosmeceutical Interest

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## Introduction

In recent years, the outward appearance and the health of the skin have become the object of interest of various scientific investigations. Research on new compounds possessing the ability to improve the condition of the skin is no longer required only for the development of new drugs, but also for cosmeceuticals – cosmetic products with scientifically proven and thoroughly tested biological activity, and therefore with higher efficiency and quality compared to traditional cosmetics available in drugstores. Considering the limited possible toxic side-effects, relatively easy synthesis, and several opportunities of further structural modifications, peptides give a fundamental contribution to the cosmeceutical field [1,2]. Collagen turnover regulation is one of the main targets for the design of new compounds relevant in the cosmeceutical arena, with particular attention on peptide-based formulas.

Among the diverse proteins in human body, collagen is one of the most essential and abundant components. Collagen type III acts as the linchpin of type I fibers and supports skin elasticity. Through complicated signaling pathway, Transforming Growth Factors-β (TGFs-β) can facilitate the biosynthesis of fibrillar collagens I and III in fibroblasts. Additionally, TGFs-β downregulate the activity of matrix metalloproteinases (MMPs), highly involved in the collagen fibers degradation [3]. The group of TGFs-β consists of five isoforms, namely TGF-$\beta_{1\text{-}5}$ [4], which function is regulated by their activation, as they exist in active or latent form (LAP-TGF-β). Secreted in the latent type, LAP-TGFs cannot react with the receptor and therefore they are biologically inert. Before the secretion by the cell, TGFs-β undergo various intracellular processes leading to their activation. *N*-terminal part of this dimer protein is called the latency associated peptide (LAP, 390 amino acid length); the *C*- terminal region is called the mature TGF-β (112 amino acid length). Various physiological substances have been found to activate the latent form of TGFs, such as: plasmin, proteases, cathepsins, calpain, and the glycoprotein thrombospondin-1 (TSP-1) (Figure 1) [5,6].

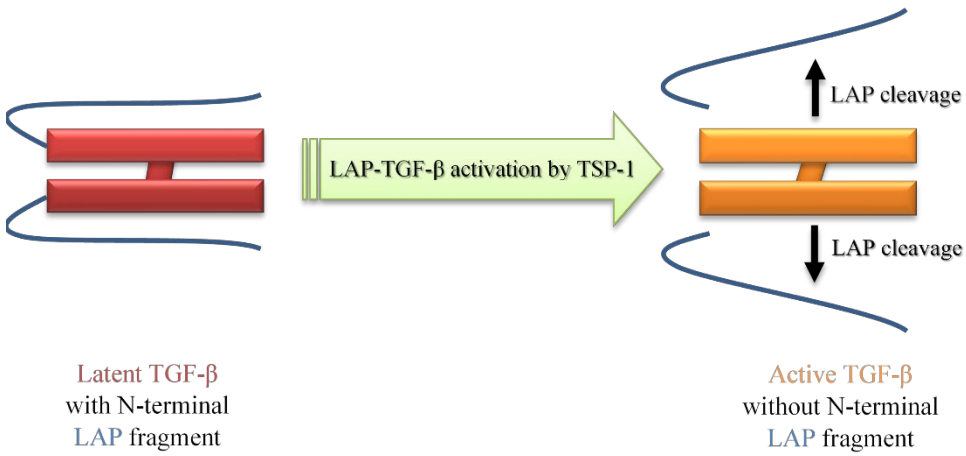


*Fig. 1. The process of latent TGF-β activation involving thrombospondin-1 (TSP-1) – its major activator in vivo.*

*Table 1. Examples of short peptides already applied in the cosmeceutical arena, with collagen synthesis-stimulating properties. They are derivatives of KRFK peptide from TSP-1, based on the initially investigated sequence KRFYVVMWKK.*

| *Sequence* | *Reference* | |
|---|---|---|
| | *Paper* | *Patent* |
| KRFYVVMWKK | Tolsma et al. 1993 [7]<br>Kanda et al. 1999 [8]<br>Denèfle et al. 2016 [9] | - |
| KRFK | Ribeiro et al. 1999 [10] | Murphy-Ullrich et al. 2002 [11] |
| KFK and Elaidyl-KFK | Cauchard et al. 2004 [12] | Bellon et al. 2001 [13] |
| RFK and KVK and their derivatives | Imfefd et al. 2015 [6] | Ziegler et al. 2007 [14] |

Latent TGF-β activation depends on the interaction of the specific TSP-1 portion $K^{412}RFK^{415}$ with LAP-TGF-$\beta_1$. This complex formation requires the interaction of TSP-1-deriving peptide KRFK and LAP-deriving fragment LSKL, located near the LAP *N*-terminus. Importantly, LAP-TSP-1 association prevents the reformation of LAP-TGF-$\beta_1$. Sequences already evaluated as activators of LAP-TGF-$\beta_1$ are summarized in Table 1.

Herein we present the KRFK peptide analogs, which will be further modified aiming at increasing the activity and, most importantly, stability and bioavailability of the original product. Our first goal, reported herein, is to evaluate the applicability of Surface Plasmon Resonance (SPR) technique in the measurements of LAP-TGF-$\beta_1$ activation process, a method not reported up to now in the literature for this type of assay. To confirm the results of this procedure, standard ELISA, widely applied for this purpose, will be performed simultaneously. Already known analogs will be synthesized as controls. Structure-activity relationship will be investigated for the sequences proving the most significant TSP-1-mimicking activity.

## Results and Discussion

**Materials and methods.** Peptides were synthesized manually *via* solid-phase peptide synthesis (SPPS) according to the Fmoc/*t*Bu strategy. ELISA assays were performed with NUNC® Maxisorp™ microplates. SPR measurements were performed with Biacore™ X100 system, for the antibody immobilization Cytiva Sensor chip CM5 was applied.

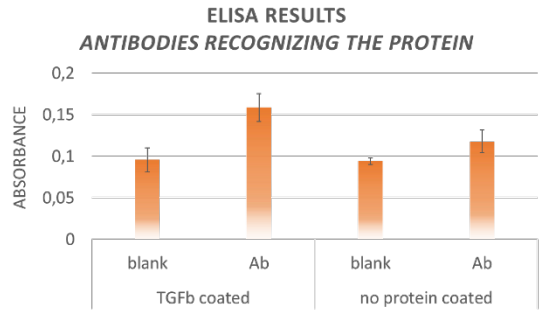


*Fig. 2. ELISA assay results confirming the activity of anti-TGF-$\beta_{1,2,3}$ antibody. Increased signal corresponds to TGF-$\beta_1$-coated wells, treated then with anti-TGF-$\beta_{1,2,3}$.*

**ELISA assays.** ELISA experiments were performed to confirm the proper activity of anti-TGF-$\beta_{1,2,3}$ antibody (MAb Clone 1D11, BioTechne) (Figure 2). For this purpose, active TGF-$\beta_1$ protein was coated on the NUNC® microplate at the concentration of 100ng/mL. After washing with saline solution, wells were blocked with a 5% Bovine Serum Albumin (BSA) in PBS solution (1.5h, room temperature). Then, anti-TGF-$\beta_{1,2,3}$ antibody was added at the concentration 5μg/mL in 2.5% BSA buffer, and incubated under agitation for 2.5h. After 3 washing cycles with saline solution, the secondary antibody (anti-mouse IgG-AP, Jackson) was added and incubated for 2h under agitation. In order to evaluate the binding of anti-TGF-$\beta_{1,2,3}$ to the protein, the microplate was developed adding the substrate solution containing 1mg/mL of *p*-NPP. The absorbance in each well was measured at 405 nm. Results of ELISA analyses are presented in Figure 3. It is visible that the

signal corresponding to TGF-$\beta_1$-coated wells, and then treated with anti-TGF-$\beta_{1,2,3}$ is the highest among all controls (two protein-coated and two non-coated wells, with and without anti-TGF-$\beta_{1,2,3}$ antibody addition in each group). Therefore, we assumed the right functioning of used antibody.

**Immobilization of anti-TGF-$\beta_{1,2,3}$ antibody onto the chip.** The standard amine coupling strategy has been chosen for the immobilization of anti-TGF-$\beta_{1,2,3}$ antibody. Sodium acetate buffer ($C_M$=50mM, pH=5) has been selected as immobilization buffer using the *pH scouting* protocol. Prior to the immobilization, the chip was activated with NHS:EDC (50:50) injection for 480s. Then, the activated chip was treated with a solution of anti-TGF-$\beta_{1,2,3}$ antibody ($C_M$=10µg/mL in immobilization buffer), by flowing it over the sensor surface for 420s at flow rate 10µL/min. The value of observed signal corresponding to the total amount of immobilized antibody was 5800 RU.

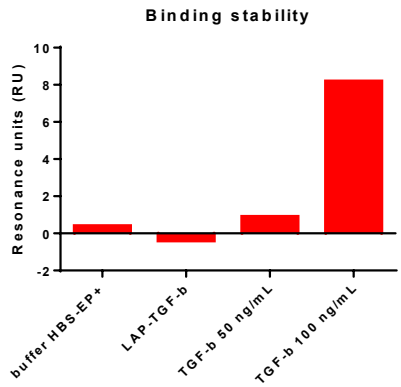


*Fig. 3. Binding stability in Resonance Units (RU) observed for different solutions of active TGF-B1 to the immobilized anti-TGF-$\beta_{1,2,3}$ antibody.*

**Selectivity of antibody immobilized on the chip.** Once anti-TGF-$\beta_{1,2,3}$ antibody was immobilized onto the sensor chip of Biacore™ X100, solutions of latent and active form of TGF-$\beta_1$ were flown through the system to prove the selectivity of chosen antibody. All proteins were tested initially at the concentration of 50ng/mL. The signals obtained for LAP-TGF-$\beta_{1,2,3}$ and active TGF-$\beta_1$ are presented in Figure 3. Due to the fact that LAP-TGF-$\beta_{1,2,3}$ do not bind to the specific immobilized antibody, and the active TGF-$\beta_1$ slightly provided a binding signal, we decided to increase the concentration of active protein solution till 100ng/mL in order to confirm the chip sensibility and selectivity. It is well visible that the signal corresponding to the blank (buffer only) is low and does not contribute significantly to observed signal intensity. Active TGF-$\beta_1$ was tested in two concentrations, 50 and 100ng/mL, and both of them were recognized by the immobilized antibody. These results proved the selectivity of used antibody, confirming that this biosensor system can be used to identify active TGF-$\beta_1$.

## Conclusions and prospective

Control sequences with already proven activity as TGF-$\beta_1$ activators (based on Table 1) were synthesized, purified, and characterized. It has been shown that inactive (LAP-conjugated) protein is not recognizable, while the signal corresponding to the free form is visible and increases with the protein concentration. ELISA assay was performed and the signal corresponding to the well with active protein and anti-TGF-$\beta_{1,2,3}$ antibody was the highest, confirming the data obtained with the Biacore™ assay.

Ongoing assays include the evaluation of the presence of free LAP, cleaved during the activation, and of the possible reassociation of LAP molecule and the activated TGF-$\beta_1$. By now it is known that the activation reaction including the full glycoprotein TSP-1 is not reversible, however there is no data clearly confirming equal behavior of shorter, TSP-1-deriving sequences. In the nearest future we aim to clarify the mechanism of LAP dissociation in the presence of synthesized peptides and to optimize the conditions of the assay involving Surface Plasmon Resonance technique.

The new protocol for Biacore™ measurements of TGF-$\beta_1$ activation, once fully optimized, will allow to perform the assay in a robust, rapid, and automated way – as compared to the time-consuming and more complicated ELISA protocols described so far.

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