

TRYLAGEN[®] PCB

CODE: P11-PD100

Date: July 2009

Revision: 4

GENERAL DESCRIPTION

Collagen is the most abundant protein in the skin, and is the major component of the extracellular matrix (ECM). Collagen makes up 70-80% of the dry weight of the skin and gives the dermis its mechanical and structural integrity [1]. The characteristic feature of a typical collagen molecule is its long, triple-stranded helical structure, in which three collagen polypeptide chains are wound around one another in a rope-like superhelix [2]. A characteristic property of collagens is to form highly organised polymers. In the fibrillar collagens (such as type I and type III collagens), they are synthesized as precursors, the procollagens; triple helices with nonhelical ends which are enzymatically removed after secretion into the extracellular space. This step is followed by fibrillogenesis in which collagen molecules assemble longitudinally head-to-tail and aggregate laterally to form collagen fibrils and fibres [3].



Fig.1. Structure of a triple-helix collagen molecule

The various collagens and the structures they form all serve the same purpose, to help tissues withstand stretching. Nineteen distinct types of collagens are recognised, all with individual characteristics that serve specific functions in a variety of tissues [2]. Some of the most important types of collagen are:

- **Type I collagen** is the most abundant collagen in the human body, representing the 80-85% of the dermal collagen. Type I collagen fibrils have a great tensile strength and elastic resistance [4,1].
- **Type III collagen** accumulates around blood vessels and is plentiful in fetal skin. Youthful skin contains a predominance of collagen III. During the aging process, cells gradually lose their ability to produce collagen type III through functional impairment and type III fibres are replaced by the stronger type I fibres [4,1].
- **Type IV collagen** is the most abundant structural component of basement membranes. Collagen IV molecules have the property to self-assemble into large polymers which constitute a stable scaffold for the basal lamina of the dermo-epidermal junction (DEJ) and an anchoring support for cells and other constituents of the basement membrane [3].

A major feature of aged skin is reduced collagen synthesis and increased degradation, resulting in connective tissue damage, and loss of the skin three-dimensional integrity [5]. Reduced synthesis of collagen types I and III is characteristic of chronologically aged skin [6]. As collagen turnover decreases during maturation, glycation of collagen occurs [7]. Glycation is a non-enzymatic reaction that takes place between free amino groups in proteins and a reducing sugar such as glucose. In skin, this reaction creates new residues or formations of cross-links (advanced glycation end products, AGEs) in the extracellular matrix of the dermis [8]. There is a marked increase of AGEs during intrinsic aging in normal human skin. Glycation of the dermis generally arises after 35 years, then increases rapidly with intrinsic aging [9]. Intermolecular cross-linking in aged skin results in the loss of elasticity and the stiffness of tissues observed during aging.

Skin aging is also associated with increased MMP (matrix-degrading metalloproteinases) expression and enhanced collagen degradation [10]. MMPs are a family of proteolytic enzymes that specifically degrade collagen, elastin and other proteins in connective tissue. MMP-2 is known to cleave type IV collagen, and is also capable of degrading type I collagen fibrils [11]. MMP-3 degrades type IV collagen of the basement membrane.

Collagen becomes less supple and more hardened with age, skin loses its elasticity and when pressed, it no longer springs back to its initial position but instead sags and forms furrows, culminating in the development of wrinkles.

The aging process can be accelerated as a result of prolonged exposure to ultraviolet (UV) radiation emanating from the sun (photoaging). Both UVA and UVB rays cause damage to collagen fibres, leading to the formation of wrinkles. Photoaged skin reveals a 20-30% decrease in type I and type III collagen within the papillary dermis. UV irradiation has been shown to decrease collagen production, and impair

organisation of collagen fibrils in skin *in vivo*. Collagen synthesis is reduced more and MMPS synthesis is greater in photoaged human skin than in naturally aged skin [12].

TRYLAGEN[®] PCB is a combination of active peptides and proteins that provide an efficient treatment to restore the collagen levels of both young and mature skin. Its activity can be described with three main functions:

A) Collagen boosting

During aging, the synthesis of collagen gradually declines. The active ingredients in TRYLAGEN[®] have proved to boost the synthesis of Collagen types I, III and IV, helping to increase the levels of collagen lost due to aging.

B) Collagen organisation

The process of fibrillogenesis results in the formation of collagen bundles that are responsible for the strength and resiliency of the skin. TRYLAGEN[®] controls collagen fibril dimensions by uniformising their diameter and regular spacing. This provides a better cohesion and stabilisation of collagen fibres, and gives suppleness to the skin.

C) Collagen protection

After a certain age the degradation of collagen speeds up, MMP levels increase, and cause the decrease of collagen in the skin. The impaired balance between collagen synthesis and degradation leads to collagen deficiency [13]. Collagenase, a neutral metalloproteinase, cleaves fibrillar collagen into specific three-quarter and one-quarter length fragments, which are then susceptible to further proteolysis [14]. TRYLAGEN[®] acts by inhibiting the production of metalloproteinases, and specifically human MMP-2 and MMP-3, therefore avoiding excessive collagen damage in aged skin. Its anti-collagenase activity protects collagen from degradation.

The modification of the dermis properties by glycation results in collagen damage. Glucose-mediated intermolecular cross-links decrease the critical flexibility of the tissues and reduce turnover [15]. TRYLAGEN[®] has also proved to inhibit glycation, avoiding the formation of AGEs, whose accumulation irreversibly induces the loss of skin elasticity.

TRYLAGEN[®] acts at three different stages in the life of collagen: synthesis, formation and degradation. The peptide combination of TRYLAGEN[™] maintains an adequate long-lasting collagen function that will ensure a healthy and youthful skin.

The benefits of TRYLAGEN[®] have also been demonstrated *in vivo*. A cream containing TRYLAGEN[®] proved to reduce significantly the depth of wrinkles around the eyes, thanks to the overall improvement in the quality of skin collagen.

PROPERTIES AND APPLICATIONS

- Selectively boosts the synthesis of Collagen I, III and IV
- Controls uniformity of fibril diameter and the regular spacing of collagen fibrils when the fibrils are formed, ensuring the correct organisation of the collagen network
- Inhibits collagen degradation and glycation characteristic of aged skin
- TRYLAGEN[®] PCB keeps our skin's collagen in balance, and this is macroscopically translated in a significant anti-wrinkle effect

TRYLAGEN[®] PCB can be incorporated in cosmetic formulations such as emulsions, gels, sera, etc., where attenuation of wrinkles is desired.

TECHNICAL INFORMATION

PRODUCT SPECIFICATIONS

Code:	P11-PD100
Appearance:	Suspension
Colour:	Yellow to Amber
Nitrogen content:	0.45 - 0.65 %
Active ingredient content:	12.5% Pseudoalteromonas Ferment Extract 2.86% Hydrolyzed Wheat Protein 1.86% Hydrolyzed Soy Protein 0.04% Tripeptide-10 Citrulline 0.01% Tripeptide-1
Preservatives:	0.71% Phenoxyethanol

PROCESSING AND DOSAGE

TRYLAGEN[®] PCB can be incorporated at the final stage of the manufacturing product, provided the temperature is below 40 °C.

It is recommended that 5% of TRYLAGEN[®] PCB is present in the final formula, in order to obtain a significant anti-wrinkle effect.

STORAGE AND SHELF LIFE

Keep in a clean, cool and dark place. If product is stored as recommended it will remain stable for at least 12 months.

SAFETY

All the materials involved are regarded as safe for their use in a cosmetic product.

EFFICACY

In vitro

A) Collagen boosting

Increase in Collagen type I synthesis

A cream containing 1.25% Pseudoalteromonas Ferment Extract was tested in an *in vitro* assay with reconstituted human skin. The variation in collagen levels was determined using the Dot Blot method on human keratinocytes on a feeder layer of 3T3 fibroblasts.

At a dose of 0.00125% Pseudoalteromonas Ferment Extract, the increase was 81% after 7 days and 128% after 15 days. (Fig. 2).

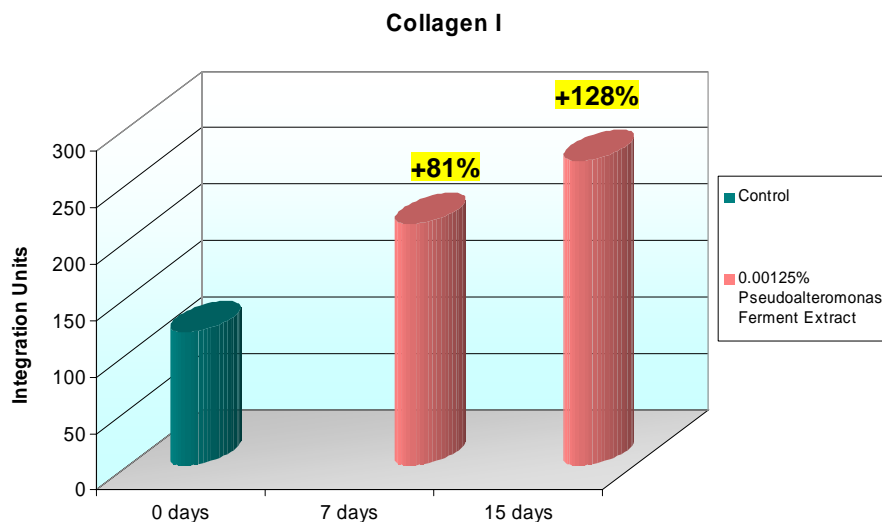


Fig. 2. Levels of collagen type I after several days of incubation with Pseudoalteromonas Ferment Extract

Increase in Collagen type IV synthesis

The same test was repeated and collagen type IV was monitored.

The increase at 0.00125% Pseudoalteromonas Ferment Extract was 36% after one week and 81% after 15 days. (Fig. 3).

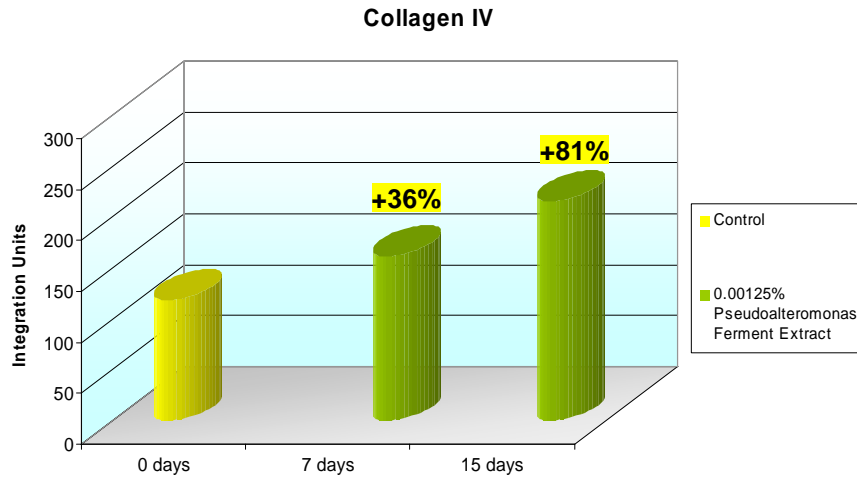


Fig. 3. Levels of collagen type IV after several days with *Pseudoalteromonas* Ferment Extract

Increase in Collagen type III synthesis

Human Dermal Fibroblasts were seeded at two different densities in 96-well culture plates and treated with a mixture of Hydrolyzed Soy Protein and Hydrolyzed Wheat Protein at different concentrations for 24 hours and 7 days. Collagen III was detected using an ELISA test with monoclonal antibodies. The increase in Collagen III can be seen even after 24 hours but a dose dependent result is obtained after 7 days. The collagen III production almost tripled at the highest dose (0.0125%) (Fig. 4).

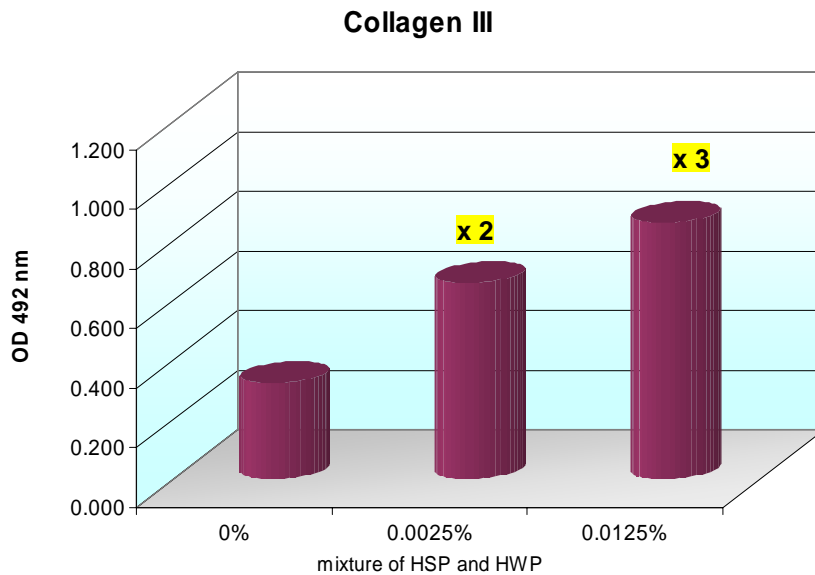


Fig. 4. Levels of Collagen Type III after 7 days at different concentrations of a mixture containing Hydrolyzed Soy Protein and Hydrolyzed Wheat Protein

B) Collagen organisation

Dermal collagen fibril study in a human skin model

Tissues from a tridimensional human skin model EFT-200 (MatTek Corporation), were treated with Tripeptide-10 Citrulline 0.01%. Non treated tissues were used as controls. Tissues were sectioned and then observed by Transmission Electron Microscopy (TEM). The diameter of collagen fibrils of two areas randomly chosen from each sample was measured.

The obtained images show that control tissues have irregular fibrils, while skin models treated with Tripeptide-10 Citrulline present more uniform collagen fibrils (Fig. 5).

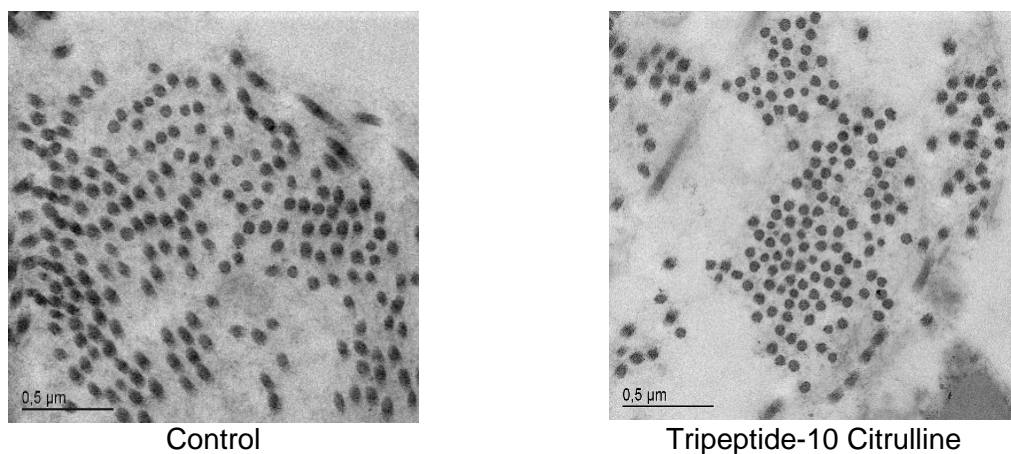


Fig.5. Transmission electron micrographs of dermal collagen from organotypic human cultures

The data obtained from the measurements was statistically analysed using the *One way ANOVA* analysis. The analysis of variances shows that the fibrils treated with Tripeptide-10 Citrulline are more uniform, due to its low variability. A 34.7% decrease of the standard deviation was observed for the fibrils treated with Tripeptide-10 Citrulline (Fig. 6).

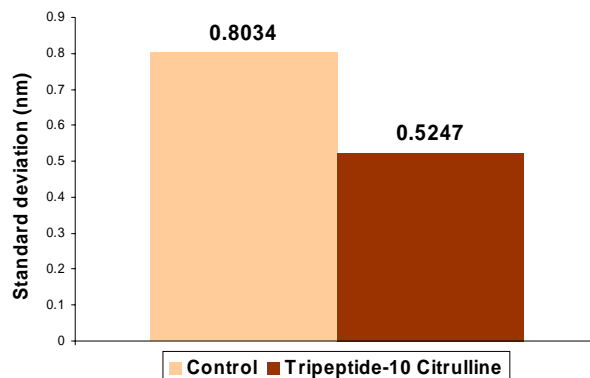


Fig. 6. Standard deviation of collagen fibril diameters in skin models ($p < 0.01$)

Also the range and distribution is different for tissues treated with Tripeptide-10 Citrulline and for control tissues. Tissues treated with Tripeptide-10 Citrulline show a perfect normal distribution with a narrower range compared to control tissues (Fig. 7).

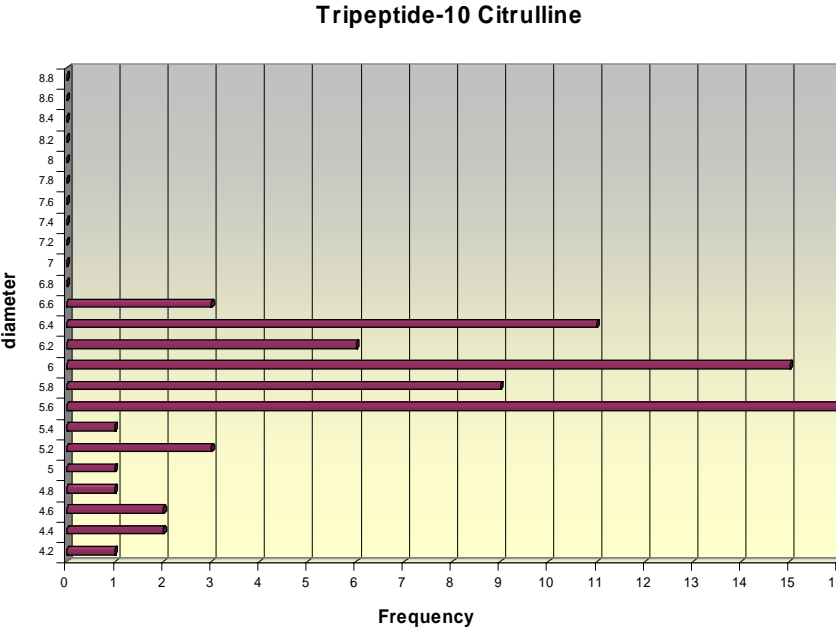
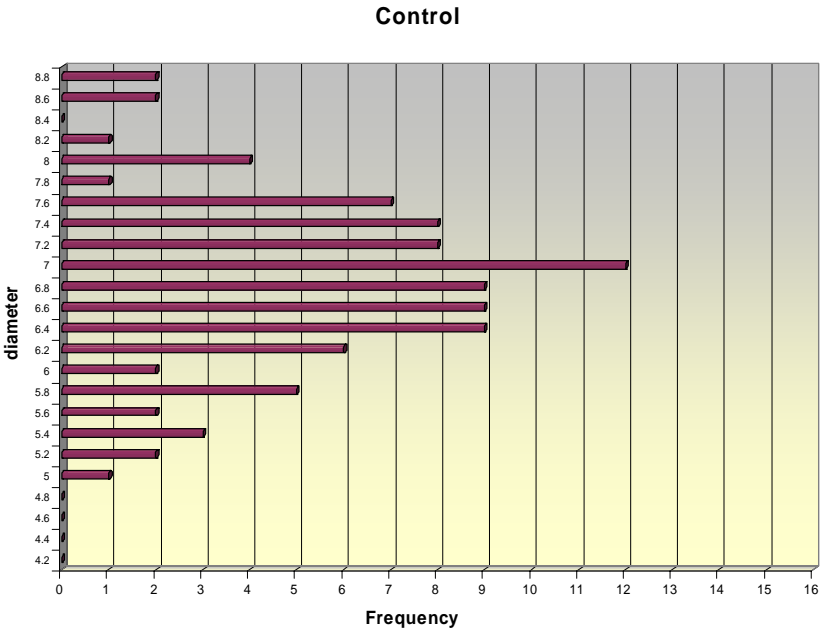


Fig. 7. Distribution of collagen fibril diameter from organotypic human cultures

C) Collagen protection

Glycation inhibition

It is known that some enzymes can suffer glycation *in vivo*. Among those enzymes we can find the Cu, Zn-Superoxide Dismutase (SOD). The SOD is an enzyme that converts superoxide radicals to hydrogen peroxide and oxygen. The incubation of SOD with glucose or other monosaccharides gives rise to glycation, which inactivates the enzyme.

In this study, the inactivation of SOD by its reaction with fructose is used as a model of glycation. The effect of Tripeptide-1 as an inhibitor of glycation is evaluated. A method to assess the SOD activity by the inhibition of the transformation of xanthine to uric acid with the enzyme xanthine oxidase is used. With this reaction, the WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) is transformed to formazan, a compound which absorbs at 470nm. If SOD is added to this reaction, radical $O_2^{\cdot -}$ is captured and the formation of this coloured compound is avoided.

The results show that there is a 39.4% increase in the SOD activity respect to controls (untreated samples) at the dose of 0.001% Tripeptide-1, which means that Tripeptide-1 inhibits SOD glycation (Fig. 8).

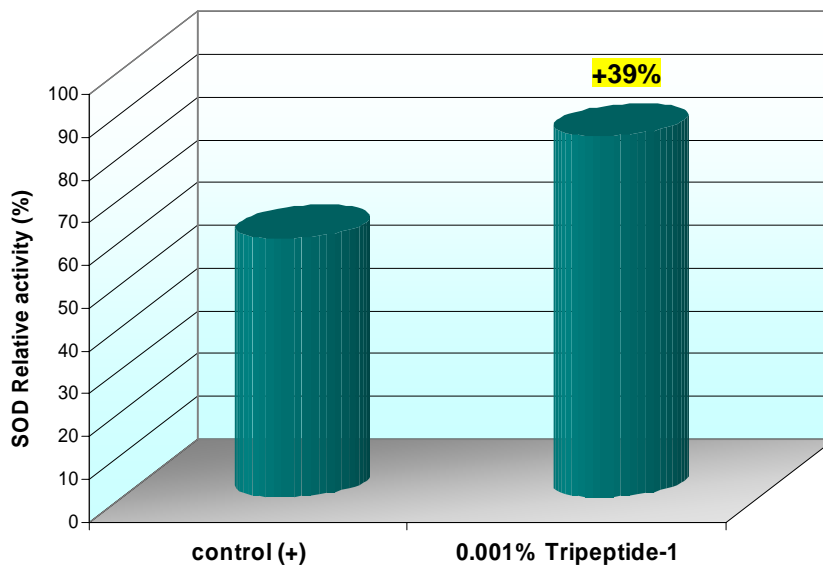


Fig. 8. Glycation inhibitory activity of Tripeptide-1

Anti-collagenase activity

Molecular Probes EnzChek Gelatinase/Collagenase Assay Kit is a high sensitivity method for measuring collagenase activity and for screening inhibitors in a high-throughput format. Collagen provided is so heavily labelled with fluorescein that the fluorescence is quenched. Collagenase type IV from *Clostridium histolyticum* served as the control enzyme. When fluorescein-labelled collagen is incubated with the enzyme and digested, it yields highly fluorescent peptides. The increase in fluorescence is proportional to proteolytic activity so a lower fluorescence demonstrates an inhibition of proteolysis. 1,10-Phenanthroline is a potent metalloproteinase inhibitor and was used as a positive control.

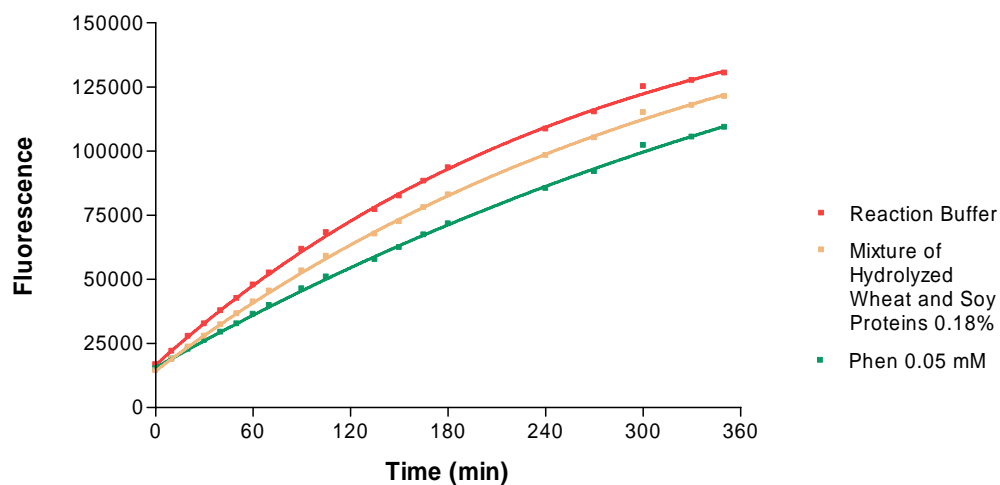


Fig. 9. Collagenase inhibition vs. time

A sample containing 0.187% of a mixture with Hydrolyzed Wheat Protein and Hydrolyzed Soy Protein showed a 13.8% inhibition of collagenase respect to control.

Human MMPs inhibition activity

The aim of this study was to determine the selectivity of TRYLAGEN[®] versus human MMPs: MMP-2 and MMP-3. The fluorescence released by quenched gelatin (denatured collagen) when digested with MMPs was monitored.

Digestion of a quenched collagenase substrate (so heavily labelled that fluorescence is quenched) and measure of released fluorescence when digested with the corresponding enzyme is a validated assay for measuring the potential inhibitory activity of compounds on the collagenase activity. The values of the fluorescence released by the digestion of labelled gelatin were corrected from the basal release in absence of MMP and TRYLAGEN[®], and normalised regarding the release of fluorescence of the control samples (untreated samples, taken as 0% inhibition).

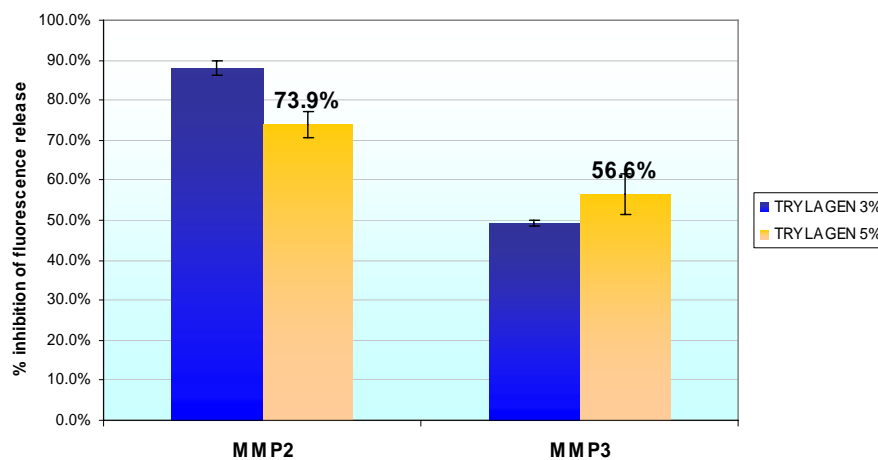


Fig. 10. Percentage of human MMPs inhibition after a treatment with different concentrations of TRYLAGEN[®]

TRYLAGEN[®] showed to inhibit the production of human MMP-2 by 73.9%, and human MMP-3 by 56.6% at the recommended dose of 5% (Fig. 10).

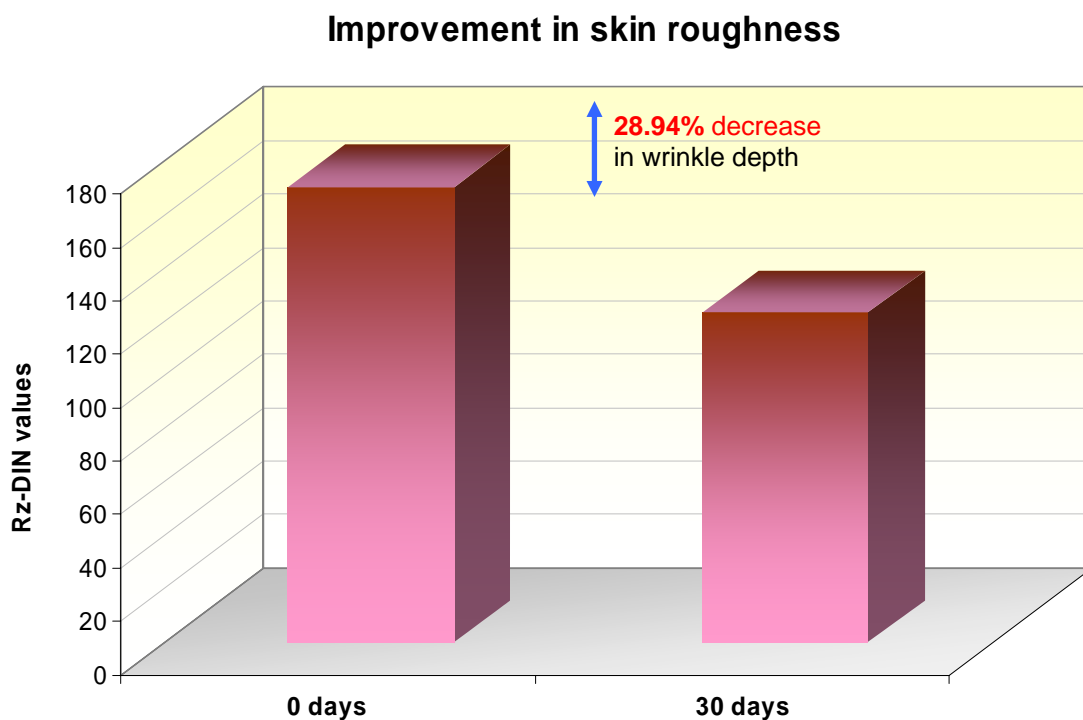
In vivo

Anti-wrinkle effect (5% TRYLAGEN®)

TRYLAGEN® has been tested *in vivo* on a group of 20 female volunteers, aged 35 to 55. A cream containing 5% TRYLAGEN® was applied twice daily on one side of the face (around the eye), and a placebo cream on the other side. The depth of wrinkles was examined before application and after 30 days by means of the optical 3D measurement PRIMOS.

In the PRIMOS measurement method, a pattern of parallel stripes is projected onto the surface of the skin and recorded by means of a system of image lenses with a CCD camera. The 3D measurement effect is achieved because the slightest differences in the height of the surface of the skin deflect the projected parallel stripes so that these deflections represent a qualitative and quantitative measurement of the profile of the skin.

The improvement in skin roughness was clearly significant for the cream containing TRYLAGEN® at the end of the test, compared to the placebo cream. A **28.94%** decrease in wrinkle depth was accomplished, with maximum values up to **-35.32%**.



Volunteer 12



0 Days



30 Days

Fig. 11. Images of Volunteer 12 before and after a 30 day treatment with a cream containing 5% TRYLAGEN®

Anti-wrinkle effect (1% TRYLAGEN®)

The same anti-wrinkle *in vivo* test was performed on a group of 20 female volunteers, aged 35 to 60. A cream containing 1% TRYLAGEN® was applied twice daily on one side of the face (around the eye), and a placebo cream on the other side. The depth of wrinkles was examined before and after 30 days of application by means of the optical 3D measurement PRIMOS.

The cream containing 1% TRYLAGEN® induced a **5% decrease in wrinkle depth** after 30 days of treatment.

GENERAL PRODUCT INFORMATION

Trade name	TRYLAGEN® PCB
Product code	P11-PD100

INGREDIENTS

INCI name	CAS No	EINECS No
WATER (AQUA)	7732-18-5	231-791-2
PSEUDOALTEROMONAS FERMENT EXTRACT	820959-16-8	N.L. ^a
HYDROLYZED WHEAT PROTEIN	70084-87-6	N.L. ^a
HYDROLYZED SOY PROTEIN	68607-88-5	271-770-5
TRIPLEPTIDE-10 CITRULLINE	960531-53-7	N.L. ^a
TRIPLEPTIDE-1	72957-37-0	277-125-4
LECITHIN	8002-43-5	232-307-2
XANTHAN GUM	11138-66-2	234-394-2
CARBOMER	9003-01-4	N.L. ^a
TRIETHANOLAMINE	102-71-6	203-049-8
PHENOXYETHANOL	122-99-6	204-589-7
BUTYLENE GLYCOL	107-88-0	203-529-7
CAPRYLYL GLYCOL	1117-86-8	214-254-7

^a Not Listed

Note: Graphs and photographs are available for customer use provided that the final product contains the same concentration of active as the formulations in our tests. Customers must request written permission for use of the graphic material and/or ingredient tradenames to Lipotec. Customers are responsible for compliance with local and international advertising regulations.

Lipotec uses the TM symbol for EU trademark applications. The symbol is changed to ® when the EU trademark is granted. The specific situation of the trademark in each country may vary and we recommend that you contact us for updated information.

REFERENCES

1. Oikarinen A. Aging of the skin connective tissue: how to measure the biochemical and mechanical properties of aging dermis. *Photodermatol Photoimmunol Photomed.* 10(2):47-52, 1994.
2. Culav EM *et al.* Connective tissues: matrix composition and its relevance to physical therapy. *Phys Ther.* 79:308-319, 1999.
3. Aumailley M & Gayraud B. Structure and biological activity of the extracellular matrix. *J Mol Med.* 76:253-265, 1998.
4. Parra JL, Pons L. *Ciencia Cosmética.* Consejo General de Colegios Oficiales de Farmacéuticos. Madrid, 1995.
5. Bernstein EF *et al.* Long-term sun exposure alters the collagen of the papillary dermis. *J Am Acad Dermatol.* 34:209-218, 1996.
6. Varani J *et al.* Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. *Am J Pathol.* 168(6):1861-1868, 2006.
7. Avery NC, Bailey AJ. The effects of the Maillard reaction on the physical properties and cell interactions of collagen. *Pathol Biol (Paris).* 54 (7): 387-395, 2006.
8. Pigeon H, Asselineau D. An *in vitro* approach to the chronological aging of skin by glycation of the collagen: the biological effect of glycation on the reconstructed skin model. *Ann N Y Acad Sci.* 1043(1):529-532, 2005.
9. Jeanmarie C, Danoux L, Pauly G. Glycation during human dermal intrinsic and actinic ageing: an *in vivo* and *in vitro* model study. *Br J Dermatol.* 145:10-18, 2001.
10. Fisher GJ *et al.* Mechanisms of photoaging and chronological skin aging. *Arch Dermatol.* 138:1462-1470, 2002.
11. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem.* 270 (11):5872-5876, 1995.
12. Chung JH *et al.* Modulation of skin collagen metabolism in aged and photoaged human skin *in vivo.* *J Invest Dermatol.* 117(5):1218-1224, 2001.
13. Varani J *et al.* Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J Invest Dermatol.* 115(7):480-486, 2000.

14. Gross J, Nagai Y. Specific degradation of the collagen molecule by the tadpole collagenolytic enzyme. *Proc Natl Acad Sci USA*. 54(4):1194-1204, 1965.
15. Paul RG, Bailey AJ. Glycation of collagen: the basis of its central role in the late complications of ageing and diabetes. *Int J Biochem Cell Biol*. 28 (12):1297-1310, 1996.