Zymomonas mobilis Levan is Involved in Metalloproteinases Activation in Healing of Wounded and Burned Tissues

Cristina Sturzoiu[1], Maria Petrescu[1], Bianca Galateanu[1], Mihai Anton[1], Cristina Nica,[1], Gheorghe (Iuri) Simionca[2], Anca Dinischiotu [1], Gheorghe Stoian[1]

[1]*Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Bucharest, 050095 - Bucharest, Splaiul Independentei, 91-95, sector 5, Romania*
[2]*National Institute for Rehabilitation, Physical Medicine and Balneoclimatology, 030077 - Bucharest, Sf. Dumitru, 2, Sector 3, Romania*

Abstract
Healing of burn tissue is a complete process involving reepitelization, granulation tissue formation and extracellular matrix remodeling. Thermal injury produces profound systemic changes, such as oligemic shock, anemia, renal failure and metabolic disorders. This causes direct tissue damages: inflammation and infection reactions. The tissue lesion also leads to increased oxidative stress in cells, as it has been observed by the low activity of endogenous antioxidant enzymatic and nonenzymatic systems. In this context, tissue matrix metalloproteinases (MMP) plays a key role in normal physiology of conjunctive tissue during its development, morphogenesis or wound healing, having an irregular activity and being involved in the patho-physiological processes. The analysis of biological samples, MMP profiles contribute to the characterization of some processes involving tissue remodeling, processes related to wound or burn healing, possibly to the development of new therapies. In this context we studied the proliferative effect of levan, a polysaccharide produced by Gram negative bacteria, *Zymomonas mobilis*, a microorganism that plays an important role in modern biotechnology to produce substances of great interest in biotechnology, food industry or in biomedicine. Our studies focused on analysis of tissue MMPs profiles from Wistar rats with lesions caused by mechanic processes on skin (wounds) and thermal (burn), treated by hallotherapy in Cacica and Dej salt mines, before and after the treatment with levan. The results indicate that levan, a natural polysaccharide produced by wild type *Z. mobilis* NCIB 11163, as well as other bacterial strains, seems to have real value in the management of wounds and burns, applied individually or in combination with natural or artificial haloteraphy. The way that levan participates in the healing process is unknown, probably by activating the tissue metalloproteinases.

Keywords: burn, levan, metalloproteinases, *Z. mobilis*, wound

1. Introduction

Chronic wounds are a huge cost to our society and health system both in terms of morbidity and expense. The American Burn Association reports half a million burn injuries per year, with 40,000 hospital admissions [1]. Matrix metaloproteinases (MMPs) play a crucial role in the tissue modeling and in tissue resorption in pathological conditions. This group of enzymes can degrade most of the proteins that compose the extracellular matrix.
MMPs are a family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related. They are secreted in an inactive (latent) form, which is called a zymogene or a pro-MMP. These latent MMPs require an activation step before they are able to cleave extracellular matrix (ECM) components [2]. MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration,

* Corresponding author: Gheorghe Stoian, Tel. 0040213181575, Fax. 0040213181575 Email: gigistoian2007@yahoo.com

differentiation, angiogenesis, apoptosis and host defense.

The activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important [3]. Overproduction of MMPs is associated with tissue destruction in chronic inflammatory diseases [4, 5]. To prevent tissue degradation by a disturbed MMP-TIMP balance, it is important to know which MMPs and TIMPs are involved in the specific disease processes. Thus, the analysis of MMPs in biological samples can contribute to the characterization of certain diseases involving tissue destruction and possibly to the development of new therapies.

In this context we studied the proliferative effect of a polysaccharide (levan, a polymer of fructose) produced by Gram-negative bacteria, *Z. mobilis*, a nontoxic product used in medicine, pharmaceutical, cosmetics and food. *Z. mobilis* is an aero-tolerant, Gram-negative and etanologenic bacterium. It is capable of producing levan as a final product of the metabolism of sucrose in concentrations up to 46%. There are well known the beneficial effects of polysaccharides in the treatment of wounds and burns, especially in traditional medicine, like honey or Aloe Vera. The aim of our studies was to analyze the tissue MMPs profiles from Wistar rats with lesions, caused by mechanic processes on skin (wounds) and thermal (burn), treated by halotherapy in Cacica and Dej salt mines, before and after the treatment with levan. The polymer was extracted and purified in our laboratory and then used in proliferation and metalloproteinase activation studies, which have a significant role in healing skin lesions at Wistar rats wounds or burns.

2. Materials and methods

Strains, growing conditions and the polysaccharide production. Cell culture of *Z. mobilis* wild type stain has been grown in solid complete medium [6, 7] at 30ºC. The extracellular polymer biosynthesized by bacteria was collected from the surface of the culture medium, with distilled water. Microorganisms were removed from the suspension by centrifugation at 1500rpm, 10 minutes, 4°C, and the polymer was precipitated with organic solvents from the supernatant [8]. Polysaccharide purity was verified by column chromatography on Biogel P200 (50-100 mesh) (Biorad Labs.) [9, 10], and the structure of levan was analyzed after acid hydrolysis in the presence of 0.05% sulfuric acid at 50°C, followed by TLC. The standard used for purity determination was fructose. The concentration of the polysaccharide was determined by spectrophotometric methods at 330nm wave length against blank [8]

Biological material. The tests were performed with whole protein extracts from normal and injured skin (burns or wounds) from Wistar rats treated by halotherapy in Cacica and Dej salt mines for two weeks. Protein concentration was determined by Lowry method [11]. MMPs presence was assessed using the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography. Gelatinolytic (MMP-2 and MMP-9) activity was shown as gel-clear zones [12].

All reagents were analytical grade. Results are the average of three independent determinations.

3. Results and discussion

Levan is a fructose polymer of microbial origin composed of β-(2,6)-fructosyl-fructose linked molecules and side chains. The molecular weight of levan obtained by microbial or enzymic synthesis is estimated to ~2.5×10^6 [13]. Levan is a major by-product synthesized of many other bacteria and also produced by *Z. mobilis*, when it is grown on sucrose. The enzyme levansucrase (EC 2.4.1.10) is responsible for sucrose hydrolysis, levan formation and oligosaccharide production. Levansucrase can be inhibited by glucose, but not by fructose, ethanol, sorbitol, NaCl or ethylenediaminetetracetate acid [14]. Figure 1 presents levan production on complete solid medium by wild type Gram-negative bacterium *Z. mobilis*.

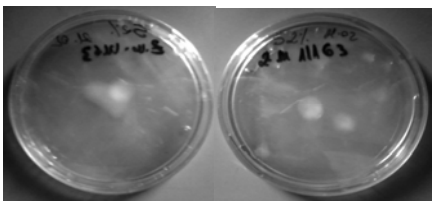

Figure 1. Levan production by *Z.mobilis* NCIB 11163 on solid medium containing 2% sucrose

Chromatographic elution was performed with saline buffer, collecting the fractions and analyzing them by spectrophotometric (Figure 2) and thin layer chromatography methods (Figure 3).

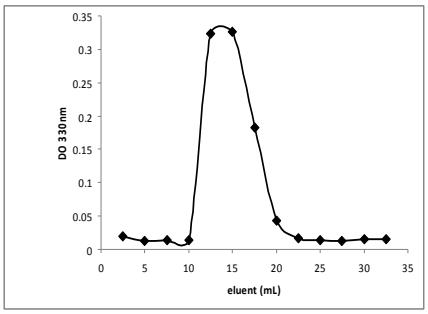

Figure 2. Levan purity determination through gel filtration chromatography on Biogel P200 column, 50-100 mesh.

Fractions absorbance values were determined at 330nm wavelengths and were plotted according to the volume of the eluent, obtaining a single peak, with maximum absorbance, demonstrating the levan presence. Sample retention time for elution was higher than those determined in the case of mesh column free space, which demonstrate that levan was retained in the three-dimensional network mesh chromatography column. Graphic results indicate that levan is the only compound eluted on Biogel P200 column (Figure 3)

Figure 3. Pointing levan fractions eluted with Biogel P-200 column, 50-100 mesh, using a TLC Silica gel 60 plates

Levan structure confirmation was achieved by compound hydrolysis in the presence of sulfuric acid 0.05%, at 50°C, taking samples every 10 minutes, and analyzing them using thin layer chromatography. The results were verified and confirmed by the standard of fructose. Levan concentration was spectrophotometricaly determined at 330nm with a value of $6x10^{-7}$mol/L, when obtained on solid medium. Protein concentration was determined by Lowry classic method. Values are lower for Wistar rats with burns and injuries, treated by speleotheraphy in Cacica salt mine, compared to control while Wistar rats two weeks old values are close to control.

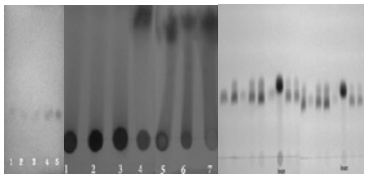

Figure 4. Results for levan hydrolysis. a. sucrose standard (mg/mL) (1-5- 1-5 µL corresponding. 10 - 50mg/10mL standard sucrose solution, b. Levan hydrolysis at 0-70 minutes, c. Levan formation in the culture medium containing sucrose, in the presence of bacteria during *Z. mobilis* fermentation.

Hydrolyze with sulfuric acid results shows that levan structure is based on fructose units. The more time hydrolysis extends, the amount of fructose increases, compared with the standard used, and weaker cluster displays of levan on chromatography. Thin layer chromatography demonstrates levan formation (left spot on the state line Silica gel 60) in culture medium containing 2% sucrose in the growing medium of *Z. mobilis* wild type stain. At the initial point of fermentation the sucrose concentration was high (2%) and after more then 25 hours, the metabolism of sucrose is more intense in the culture medium with formation of levan, fructose and other secondary compounds.

The metalloproteinases tissue profiles were examined in Wistar rats with lesions caused by mechanical procedures (wounds) and thermal (burn) on skin treated by halloterapy for two weeks in Cacica and Dej salt mines. Figures 5 and 6 show tissue metalloproteinases (gelatinase activity) of protein extract obtained from Wistar rat skin burned and injured. Our studies have focused on metalloproteinase profiles for analysis after treatment with levan.

To understand the underlying mechanisms involved in pathological conditions such as fibrosis and chronic non-healing ulcers, it is helpful to review first the knowledge about normal tissue response to injury [15, 16]. Healing of burned tissue is a complex process, which involves re- epithelisation, granulation tissue formation and remodeling of extracellular matrix. There is also experimental evidence that indicates the involvement of superoxide radical in the pathogenesis of burn wound [17].

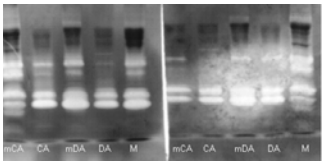

Figure 5. Gel electrophoresis (SDS-PAGE) for highlighting metalloproteinases tissue (A) biological samples treated by halotherapy and MMP levan (6 x 10^{-7} mol/L) activation. Legend: MCA- burned Wistar rats two weeks old treated in Cacica salt mine, CA – burned adults Wistar rats treated in Cacica salt mine, MDA- burned adults Wistar rats tow weeks old treated in Dej salt mine; DA- burned adults Wista rats treated in Dej salt mine, M-adults normal Wistar rats (control); (B) MMP with simple activation.

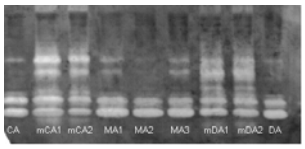

Figure 6. Gel electrophoresis (SDS-PAGE) for highlighting metalloproteinases tissue , DR- wounded adults Wistar rats treated in Dej salt mine, MR- wounded adults Wistar rats with any tratament, M- adults normal Wistar rats (control)

Various biological and metabolic changes occur during burn injury including degradation of adenosine triphosphate (ATP) and significant reduction in polyunsaturated fatty acids in red cell membrane [18]. Free radicals as well as histamine and prostaglandins released from the burn wound cause lipid peroxidation in the skin tissue. These radicals appear to be involved in the appearance of lipid peroxidation products extractable from burned skin and distant organs [19, 20, 21, 22, 23]. Wound healing involves a complex interaction between epidermal and dermal cells, the extra cellular matrix, controlled angiogenesis and plasma- derived protein all coordinated by an array of cytokines and growth factors [24]. MMP play an important role in the tissue remodeling occurring during physiological or pathological processes [25].

The pathologic mechanisms underlying why some wounds heal well and some poorly are only partially understood. Matrix metalloproteinase type 9 (MMP-9) is a collagenase that is associated closely with chronic diseases such as cancer and chronic wounds [22, 26, 27, 28, 29, 30]. MMP-9 is persistently elevated in chronic wounds and in increased ulcers and venous stasis ulcers. The MMP-9 concentration inhibition associated with delays in keratinocyte motility in vitro, delayed epithelialization in vivo, and delayed angiogenesis [16, 31, 32, 33,]. A critical role of MMP-2 and MMP-9 has been also shown for the development of abdominal aortic aneurysm using MMP gene deletion mice [34]. Both MMP-2 and MMP-9 cleave elastin, type IV collagen, and several other ECM molecules and MMP-2 digests interstitial collagen types I, II and III. MMP-9 is not expressed in normal, uninjured skin, while MMP-2 is constitutively expressed at minimal levels [35, 36]. In some studies on regulation of elastase-type endopeptidase activity, showed that MMP2 and MMP9 are more expressive and activated in human dermal by fucose and a fucose-rich polysaccharide. Studies showed that mono-, di-, oligo- and polysaccharides acting on the elastin-laminin receptor and/or on the fucose-mannose receptor are efficient inhibitors of such enzymes by down regulating elastase-type endopeptidase activity, both at the level of their biosynthesis and at the level of the activation of the pro-enzymes. Fucose and fucose-rich polysaccharide preparations were shown to be efficient modulators of MMP-2 and MMP-9, activity with potential therapeutic applications in age-related pathologies accompanied by tissue loss [37]. Integrins have been found to play a role in platelet aggregation, immune functions, tissue repair, and tumor invasion, and some diseases are seldom known to be caused by mutations in integrin genes. There is increasing evidence that the cell movements that take place during tissue repair such as wound healing depend on integrin-mediated interactions [38].

The analysis of biological samples MMP may contribute to the characterization of processes involving tissue remodeling, processes related to wound healing or burn, possibly to the development of new therapies.

Metalloproteinases activation by levan occurs also when Wistar rats were not treated by thermic burns and injury (data no shown).

4. Conclusions

Our results showed an intense metalloproteinases expressions present in the skin, through the activation with polysaccharides, less or never used until now in treating wounds and burns, using Wistar rats experimental models, treated by speleotherapy/haloterapy in salt mines.

Biochemical analysis indicates a significant activation of MMP-2 and MMP-9 respectively, after levan treatment in combination with speleotherapy for two weeks. This is particularly important in preventing infections in thermal or mechanical injuries occurring above epidermal tissue and celular epithelial proliferation, as well as speeding up efficiently healing of wounds and burns. Our studies indicate that levan therapy could provide a new and valid option, with results significantly beneficial for skin injuries *in vitro* and *in vivo*. Although in this direction are not very rich information, so presumably that mechanisms are closely related to cellular proliferation, protection systems against oxidative stress, metalloproteinases overexpression being involved in recovery, reconstruction and epithelium regeneration cell systems.

Acknowledgements

This work was supported by project ZYSOPROD, PN II62-068/2008 and CEFACTERMEDSUB, PN II 42-160/2008. Cristina Sturzoiu thanks to project POSDRU/88/1.5/S/61150 "Doctoral Studies in the field of life and earth sciences", project co-financed through Sectorial Operational Program for the Development of Human Resources 2007-2013 from European Social Fund for financial support.

References

1. Heit, J. A., Rooke, T. W., Silverstein, M. D., Mohr, D. N., Lohse, C. M., Petterson, T. M., O'Fallon, W. M., Melton, L. J., 3rd. Trends in the incidence of venous stasis syndrome and venous ulcer: a 25 year population based study, Journal of Vascular Surgery, 2001, 33, 1022–1027

2. Bode, W., Maskos**,** K., Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases, Biological Chemistry, 2003, 384, 863-872

3. Brew, K., Dinakarpandian, D, Nagase, H., Tissue inhibitors of metalloproteinases: evolution, structure and function, Biochimica et Biophysica Acta, 2000, 1477, 267-283

4. Konttinen, Y. T., Ainola M., Valleala H., Ma J., Ida H., Mandelin J., Kinne R. W., Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis, Annals of the Rheumatic Diseases, 1999, 58:691-697

5. Tetlow, L. C., Adlam D. J., Woolley D. E., Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes, Arthritis & Rheumatism, 2001, 44, 585-594.

6. Calazans, G. M. T., Lima, R. C., de França, F. P., Lopes, C. E., Molecular weight and antitumour activity of *Zymomonas mobilis* levans, International Journal of Biological Macromolecules, 2000, 27, 245-247

7. Galani I., Drainas, C., Tipas, M. A., Growth requirements and the establishment of a chemically defined minimal medium in *Z. mobilis,* Biotechnology Letters, 1985, 7, 9, 673-678

8. Andersone, I., Auzina, L., Mutere, O., Zikmanis, P., Formation of levan from raffinose by levansucrase of *Zymomonas mobilis*, Engineering in Life Sciences. 2004, 4, 56-59

9. Reese, E. T., Avigada, G., Purification of levansucrase by precipitation with levan, Biochimica et Biophysica Acta (BBA) - Enzymology and Biological Oxidation, 1966, 113, 1, 79-83

10. Rees, R. S., Smith, D. T., Li, D., Cashmer, B., Garner, W., Punch, J., Smith, Jr. D. J., The role of xanthine oxidase and xanthine dehydrogenase in skin ischemia., Journal of Surgical Research, 1994, 56,162–167.

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. ., The Journal of Biological Chemistry, 1951, 193: 265-275

12. Moldovan, L., Cimpean, A., Zarnescu, O., Flamanzeanu, M., Nicolae, C., Dumitrescu, D., Matrix Metalloproteases Involved in Injured Corneal Tissue Remodelling, Roumanian Biotechnological Letters, 2002, 7, 6, 995-1002

13. Iizuka, M., Yamaguchi, H., Ono, S., Minaminra, N., Production and isolation of levan by use of levansucrase immobilized on the ceramic support Sm-10, Bioscience, Biotechnology, and Biochemistry, 1993, 57, 322–324.

14. Crittenden, R. G., Doelle, H. W., Identification and characterisation of the extracellular sucrases of *Zymomonas mobilis* UQM-2716 (ATCC-39676), Applied Microbiology and Biotechnology, 1994, 41, 302–308

15. Rees, R. S., Smith, Jr., D. J., Adamson, B., Im, M., Hinshaw, D., Oxidative stress: the role of the glutathione redox cycle in skin preconditioning, Journal of Surgical Research, 1995, 58, 395–400.

16. Scott, K. A, Arnott, C. H., Robinson, S. C., Moore, R. J., Thompson, R. G., Marshall, J. F., TNF-alpha regulates epithelial expression of MMP-9 and integrin alphavbeta6 during tumour promotion. A role for TNF-alpha in keratinocyte migration, Oncogene, 2004, 23, 6954–6966

17. Cetinkale, O., Konukoglu, D., Penel, O., Kemerli, GD., Yazar, S., Modulating the functions of neutrophils and lipid peroxidation by FK 506 in a rat model of thermal injury, Burns, 1999 , 25, 105–112

18. Hertford, C., A. E. Cream,. Thermal injuries. In: Synopsis of surgery, Liehty, R.D. Eds. 4th edn, C.V. Mosby Co, 1980, 407

19. Demling, R. H., Katz, A., Lalonde, C., Ryan P., Jin L.J., The emmediate effect of burn wound excision on pulmonary function in sheep. The role of prostanoids, oxygen free radical and chemoattractants, Surgery, 1987, 101, 44-55
20. Nishigaki, L., Hagihara M., Hiramatsu M., Izawa Y., Yagi K., Effect of thermal injury on lipid peroxidation level in rat, Biochemical Medicine, 1980, 24, 185-189
21. Till, G. O., Hatherill, J. R., Tourtellotte, W. W., Lutz M. J., Ward, P. A, Lipid peroxidation and acute lung injury after thermal trauma to skin. Evidence of a role for hydroxyl radical, The American Journal of Pathology, 1985, 119, 376-384
22. Sturzoiu, C., Constantinescu, A. A., Hoteteu, M., Simionca, I. (G)., Stoian, G., Studies on the effect of experimental speleotherapy cure in salt and cave cavities designated with therapeutic potential properties by identifying the biochemical stress mechanisms involved in allergic disease in Wistar rats, National Conference of Balneology VIII, Bucharest, 5-6 November, 2010, pp. 5
23. Thomson, P. D., Till, G. O., Woolhscrot, J. O., Superoxide presents lipid per oxidation in burned patient, Burns, 1990, 16, 406-413
24. Bhat, R. S., Shankrappa, J., Shivkumar, H. G., Formulation and evaluation of poly herbal wound treatments, Asia Journal of Pharmacology Sciences, 2007, 2, 171-172
25. Pillai, S., Oresajo, C., Hayward, J., Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation induced matrix degradation - a review, International Journal of Cosmetic Science, 2005, 27, 17-34
26. Kaomongkolgit, R., Cheepsunthorn, P., Pavasant, P., Sanchavanakit, N., Iron increases MMP-9 expression through activation of AP-1 via ERK/Akt pathway in human head and neck squamous carcinoma cells, Oral Oncology, 2008, 44, 587–594
27. Vina, I., Karsakevich, A., Bekers, M., Stabilization of anti-leukemic enzyme L-asparaginase by immobilization on polysaccharide levan, Journal of Molecular Catalysis B: Enzymatic, 2001, 11, 551-558
28. Weckroth, M., Vaheri, A., Lauharanta, J., Sorsa, T., Konttinen, Y. T., Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers, Journal of Investigative Dermatology, 1996, 106, 1119-1124
29. Wysocki, A. B., Staiano-Coico, L., Grinnell, F., Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9, Journal of Investigative Dermatology, 1993, 101, 64–68
30. Yager, D. R., Zhang, L. Y., Liang, H. X, Diegelmann R. F, Cohen, I. K., Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids, Journal of Investigative Dermatology, 1996, 107, 743–738
31. Agren, M. S, Mirastschijski U, Karlsmark, T., Topical synthetic inhibitor of matrix metalloproteinases delays epidermal regeneration of human wounds, 2000, Experimental Dermatology, 10, 337–348
32. Colnot, D. R, Roos J. C, de Bree R, Wilhelm A. J, Kummer J. A, Hanft G, Heider K. H, Stehle G, Snow G. B, van Dongen G. A., Safety biodistribution, pharmacokinetics, and immunogenicity of 99mTc-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with squamous cell carcinoma of the head and neck, Cancer Immunology, Immunotherapy, 2003, 52, 576–582
33. Moses, M. A., The regulation of neovascularization of matrix metalloproteinases and their inhibitors, Stem Cells, 1997, 15, 180–189
34. Longo, G. M., Xiong, W., Greiner, T. C., Zhao, Y., Fiotti, N., Baxter, B. T., Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms, European Journal of Clinical Investigation, 2002, 110, 625–632
35. Tang, C. H., Tan, T. W., Fu, W. M., Yang, R. S., Involvement of matrix metalloproteinase-9 in stromal cell-derived factor-1/CXCR4 pathway of lung cancer metastasis, Carcinogenesis, 2008, 29, 35–43
36. Yuecheng, Y., Xiaoyan, X., Stromal-cell derived factor-1 regulates epithelial ovarian cancer cell invasion by activating matrix metalloproteinase-9 and matrix metalloproteinase-2, European Journal of Cancer Prevention, 2007, 16, 430–435
37. Isnard, N., Péterszegi, G., Robert, A. M., Robert, L., Regulation of elastase-type endopeptidase activity, MMP-2 and MMP-9 expression and activation in human dermal fibroblasts by fucose and a fucose-rich polysaccharide, Biomedicine & Pharmacotherapy, 2002, 56, 258-264
38. Clark, R. A. F., Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin, Journal of Investigative Dermatology, 1990, 6, 128S-134S