

Streaming of Proteolytic Enzyme Solutions for Wound Debridement: A Feasibility Study

Tali Yaakobi;¹ Dalit Roth;¹ Yoram Chen;² Amihay Freeman¹

AUT: Please provide author credentials e.g., MD? PhD?, BS?

From the ¹Department of Molecular Microbiology and Biotechnology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel; ²Enzysurge Ltd., Misgav, Israel

Abstract: The effective enzymatic digestion of extracellular matrix for the preparation of cell cultures paved the way to its application for skin treatment and wound debridement, encouraged by the selectivity observed for the separation of dermis from epidermis or the removal of necrotic tissue from wound bed without damage to healthy tissue. Proteases, such as papain and collagenase, mostly formulated as ointments, were successfully employed for wound debridement, achieved within several days of repeated treatments. Here, we propose and provide feasibility demonstration of a new mode of enzyme application for skin treatment and wound debridement: continuous controlled streaming of enzyme solutions onto an enclosed treated area. The working hypothesis is that the combination of fresh supply of enzymes in optimal working buffer with continuous flow will substantially shorten the time required for effective treatment, e.g., from days or weeks to hours, as well as simplify handling and processing. The feasibility of enzyme streaming and its efficacy are demonstrated in targeted selective intact skin digestion, removal of coagulated blood, and debridement of experimental burn wounds in lab animals. Selective skin digestion and burn wound debridement with minimal handling were readily achieved within 2 to 3 hours by streaming diluted protease solutions at slow flow rates. This simple and straight-forward mode of operation carries potential for the improvement of wound bed management. The use of this streaming technique may facilitate delivery of cleaning solutions, enzymatic debriders, or irrigation fluids for wound bed preparation.

WOUNDS 2004;16(6):193-197

E nzymatic digestion of the intercellular matrix for the separation of mammalian cells is a common methodology in the preparation of primary cell cultures.¹ This procedure, resulting in suspended isolated cells, has been routinely employed for the preparation of cell cultures from a wide spectrum of organs, including skin.² Some of the proteases used in these procedures were demonstrated to be selective in disruption of the extracellular matrix and adhesion proteins without causing cell damage.^{3,4} Further-

more, separation of dermis from epidermis^{2,4,5} and the removal of necrotic or damaged tissue from wounds and burns without damage to healthy tissue were also demonstrated.^{6,7} These observations paved the way to systematic exploration of the potential inherent in wound debridement by enzymes. Most of these studies employed commercially available enzymes (e.g., papain, bromelain, collagenase, trypsin, thermolysin) with only few attempts to identify or develop new enzymes, e.g., from the antarctic krill.⁶⁻⁸

Address correspondence to:

Prof. Amihay Freeman, Department of Molecular Microbiology and Biotechnology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978 Israel

Phone: 972-3-640-9054; Fax: 972-3-640-5147; E-mail: amihayf@post.tau.ac.il

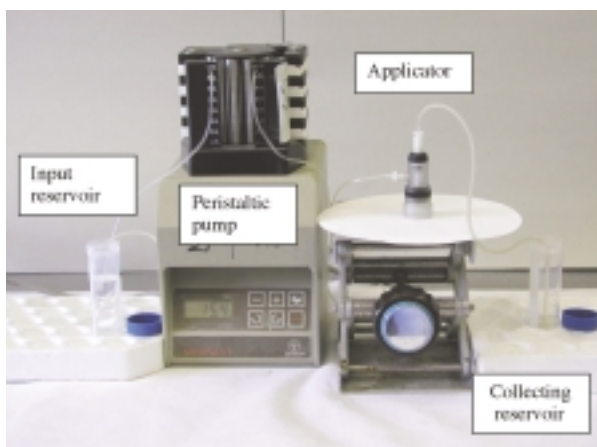


Figure 1. Shown here are the components of an enzyme streaming system.



Figure 2. This photograph demonstrates simultaneous six-channel streaming of enzymes.

Enzymes tested for effective wound debridement were mostly formulated as ointments, solutions absorbed by a wet gauze, hydrocolloids, or hydrogels.^{3,6,7} Comparative studies on the efficacy of wound debridement by enzyme-containing ointments—either animal models or humans—indicated efficacy dependence on the enzyme employed: While fibrinolysin ointment was found ineffective, collagenase ointment gave some improvement, and papain-urea ointment was identified as most effective from this group.⁹⁻¹² Prolonged time was required for these treatments to deliver significant improvement, ranging from four days to three weeks with daily wound treatments for fresh ointment supply (for the first 4–7 days of treatment).⁹⁻¹²

The objective of this study was to test the feasibility of a new mode of enzyme delivery for skin treatment and wound debridement—continuous controlled streaming of enzyme solutions onto the targeted treated area. The working hypothesis was that the combination of a fresh supply of enzymes in an optimal working buffer with continuous flow would substantially shorten the time required for effective

enzymatic skin treatment or wound debridement. Furthermore, the slightly pressurized stream would allow homogeneous supply of the enzyme to all parts of the treated area and may remove cells, debris, and solubilized proteins.

In this report, the feasibility of enzyme streaming and its efficacy was studied regarding intact skin digestion, removal of coagulated blood, and debridement of experimental surgical and burn wounds.

Materials and Methods

The streaming system was comprised of a feeding reservoir, connecting tubing, peristaltic pump (MP4 Minipulse 3, Gilson, France), a plastic applicator designed to direct the flow onto the treated site, and collecting vessel (Figure 1).

Animals and tissue samples. The study was performed on groups of six 4- to 8-weeks-old (30–40g body weight) male and female white mice, on groups of six mature 2- to 3-months-old (200–250g body weight) Charles-River male rats, on one adult male New-Zealand white (NZW) rabbit (3kg body weight), and on pig skin samples freshly removed from a large, white, male pig (34kg body weight) (**AUT: include age of pig?**). The mice and rats were anesthetized with 0.1mL of 1.25-percent tribromoethanol saline per 10g body weight, and the rabbit was sedated by ketamine and anaesthetized with thiopentone sodium. The areas to be treated on all animals were shaved, and the animals were placed on a jack and lifted until the applicator was tightened to the surface of the postero-lateral aspect of their backs. The fresh pig skin samples were mounted on a plastic O-ring and then fastened to the applicator.

Enzymes. All enzymes tested were lyophilized powders supplied by Sigma (Sigma-Aldrich Chemicals, St. Louis, Missouri, USA). The enzymes were utilized as received without further purification. The effects of the following enzymes were investigated: bromelain (B4882, dissolved in 0.01M Tris [T1503, Sigma] pH7.5); collagenase (C01300, dissolved in 0.1M Tris pH7.6); papain (P4762, dissolved in 0.01M phosphate buffer pH6.5 containing 5mM L-Cystein [16,814-9, Sigma] and 2mM ethylenediaminetetraacetic acid [EDTA] [E9884 Sigma]); pepsin (P7012, dissolved in 10mM HCl pH 2.9); protease type X (Thermolysin, P1512 dissolved in 10mM sodium

acetate [TA948368, Merck] and 5mM calcium acetate [C1000, Sigma]); and trypsin (T1005, dissolved in 0.01M Tris pH8.6).

Intact skin treatment. Freshly prepared solutions were continuously streamed onto confined shaved skin surface areas of the anesthetized mice, rats, rabbit, or pig skin samples at a flow rate of 5 to 6mL/hour for three hours at room temperature, after which the animals were sacrificed and samples for histological examination were removed from treated areas.

Histology. Following the three hours treatment, the mice and rats were sacrificed with an overdose of chloral hydrate (Fluka Chemicals, Switzerland). and the rabbit was sacrificed with an overdose of thiopenton sodium. Full-thickness skin samples (4x15mm) were removed for histological analysis from the margins of the confined area to allow comparison of treated and nontreated areas in same slide. Tissue samples were immediately fixed in four-percent phosphate buffered formaldehyde for 48 hours, processed by routine histological procedures, and embedded in paraffin. Serial sections perpendicular to the skin surface were cut at 8_ thickness (**AUT: 8_ did not translate. Pls clarify**). The sections thus obtained were stained with hematoxylin and eosin for observations.

Experimental wound models. Thermal burns, 1 to 1.5mm in depth, were induced by a direct contact of a tip of a standard soldering instrument for 30 seconds on the postero-lateral aspect of dorsal skin of anesthetized mice and rats as previously described.¹⁰ Freshly prepared single or combination protease solutions were applied by continuous streaming onto the wound within one hour from injury for 2 to 3 hours at the same flow rate as mentioned above.

Full-thickness linear fresh cuts were made by scalpel on the postero-lateral aspect of animal backs and immediately treated with continuous streaming of enzymes for three hours.

Photographs of treated areas were taken immediately after treatment and after seven and 20 days for assessment of the healing process.

Monitoring of streamed enzymatic activity. As proteolytic enzyme solution may lose proteolytic activity due to autodigestion, residual activity of enymes employed was routinely monitored by *in-vitro* biochemical assays recommended by the supplier.

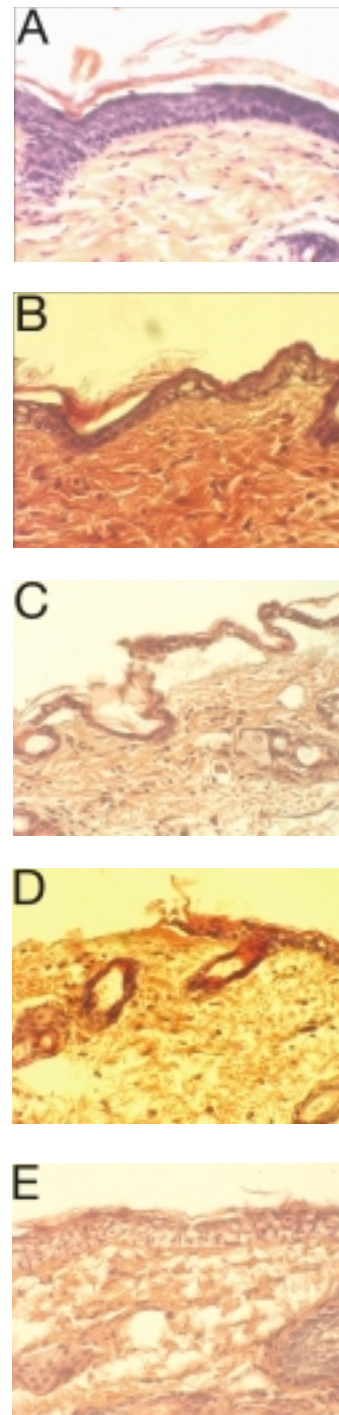


Figure 3. Pictured here are histological sections of mouse skin treated with streaming of proteases for three hours: (A) untreated; (B) papain treated (right side); (C) trypsin—bromelain mixture; (D) trypsin (left side); (E) pepsin.

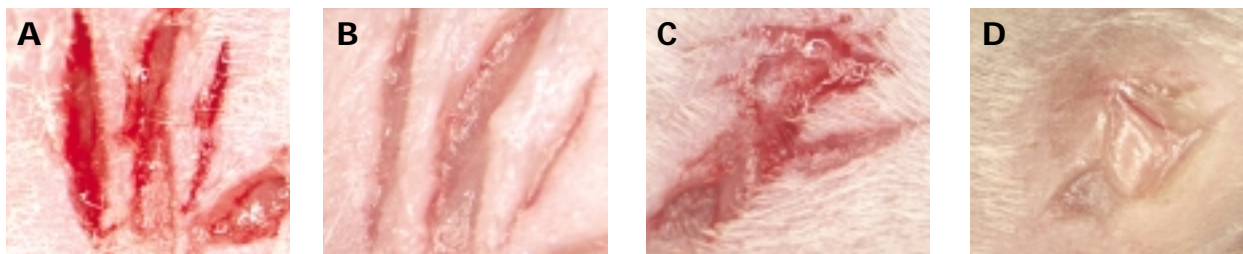


Figure 4. These photographs demonstrate effectiveness of removal of coagulated blood by streaming of trypsin and collagenase mixture.

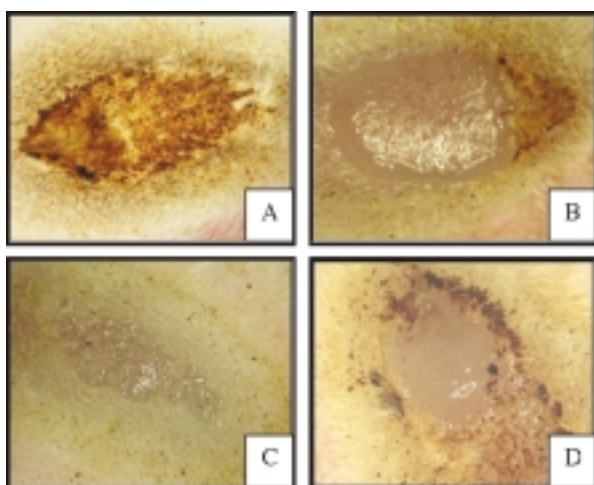


Figure 5. These photographs illustrate burn wound debridement by streamed proteases: (A) untreated; (B) collagenase/thermolysin treated; (C) trypsin/papain treated; (D) trypsin/collagenase treated.

Results

Effect of enzyme streaming on intact skin.

Controlled streaming of enzymes could be readily and conveniently applied as a series of consecutive treatments using a multichannel pump, as demonstrated in Figure 2A, for treatments of six anesthetized rats or treatment of six different sites on a larger animal (Figure 2B). Effective digestion of different skin layers was readily achieved by streaming diluted buffered enzyme solutions for three hours. The controlled streaming of 2mg/mL papain onto mice effected digestion and removal of the outer keratinized layer (compare Figure 3A to Figure 3B). Detachment of the epidermis from the dermis was effected by a trypsin (4mg/mL) and bromelain (5mg/mL) mixture (Figure 3C). Controlled streaming of 8mg/mL trypsin solution

effected complete digestion of the epidermis layer (Figure 3D). Streaming of 3mg/mL pepsin resulted in deeper penetration and collagen fiber digestion (Figure 3E). Streaming of a mixture of 3mg/mL collagenase and 1.5mg/mL thermolysin resulted in digestion similar to that shown in Figure 3D.

Similar results were obtained by streaming of same solutions on rat, rabbit, and pig skins (data not shown).

Streaming of active enzyme solutions was essential to obtain these effects. Streaming of buffer solution without enzymes was ineffective. Furthermore, streaming of enzyme solution for a few minutes to fill the system followed by flow arrest also resulted in no visual change.

The specific activity of all streamed enzyme solutions remained stable (>85%) throughout the three-hour application period. The minor loss of input activity was most probably caused by autodigestion.

Effect of enzyme streaming on experimental wounds. Effective removal of fresh blood clots was readily achieved by streaming of trypsin and collagenase mixture (3mg/mL each) for three hours onto freshly made cuts with smooth surface cleaning regardless of their shape (compare Figures 4A and C with Figures 4B and D).

Controlled enzymatic streaming for burn wound debridement was also readily achieved by two hours streaming of several protease combinations, including collagenase/thermolysin mixture (3mg/mL and 1.5mg/mL, respectively) (Figure 5B), trypsin/papain mixture (4mg/mL and 2mg/mL) (Figure 5C), and trypsin/collagenase mixture (3mg/mL each) (Figure 5D).

Debridement with streamed enzymes, e.g., papain or pepsin (2mg/mL and 3mg/mL, respectively), for two hours resulted in smooth healing

(compare Figure 6A with Figure 6B; photographs taken 20 days post-burn induction).

Discussion

The overall objective of this study was feasibility and efficacy demonstration of enzyme streaming for skin treatment and wound debridement as an alternative and highly effective mode of enzyme application. The working hypothesis was that continuous streaming will minimize handling and provide higher efficacy and more rapid debridement. The continuous fresh supply of the enzyme dissolved in solutions buffered for optimal activity would result in effective distribution and access to all parts of the treated area as well as in better control of the process. Moreover, the continuous stream could help in washing away digested material resulting in faster erosion and a shortened procedure. This flow system may also allow step-by-step treatment with different solutions, such as pretreatment with antibiotics, enzymatic digestion, and wound cleaning.

The feasibility of this approach was demonstrated on lab animals by studies on skin treatment and wound debridement. Our results have clearly demonstrated technical feasibility and efficacy of streaming of enzyme solution. The time required for effective treatment was on a scale of few hours, substantially shorter than the several days/weeks required for treatment with enzyme-containing ointments.

The simplicity of this method carries potential for wound bed management by the sequential streaming of solutions for softening, cleaning, debriding, and washing wounds in order to prepare them for optimal healing or subsequent procedures.

References

1. Ferkushny RI. *Culture of Animal Cells*. New York, NY: AR Liss, 1983:108.
2. Hybbinette S, Bostrom M, Lindberg K. Enzymatic dissociation of keratinocytes from human skin biopsies for *in-vitro* cell propagation. *Exp Dermatol* 1999;8:30-8.
3. Berger MM. Enzymatic debriding preparations. *Ostomy Wound Manage* 1993;39:61-9.
4. Normand J, Karasek MA. A method for the isolation

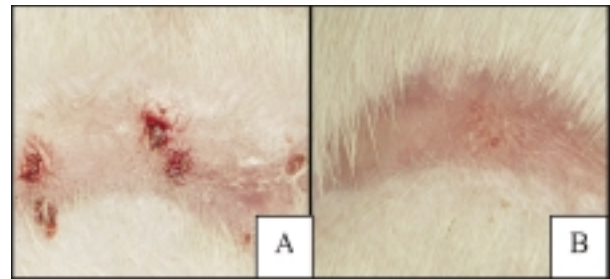


Figure 6. These photographs illustrate healing of burn wounds debrided by papain: (A) untreated; (B) papain treated.

- and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In-Vitro Cell Dev Biol Anim* 1995;31:447-55.
5. Germain L, Guignard R, Rouabhia M, Auger A. Early basement membrane formation following the grafting of cultured epidermal sheets detached with thermolysin or dispase. *Burns* 1995;21:175.
6. Falanga V. Wound bed preparation and the role of enzymes: A case for multiple actions of therapeutic agents. *WOUNDS* 2002;14:47-57.
7. Klasen HJ. A review on the nonoperative removal of necrotic tissue from burn wounds. *Burns* 2000;26:207-22.
8. Mekkes JR, LePoole IC, Das PK, Bos JD, Westerhof W. Efficient debridement of necrotic wounds using proteolytic enzymes derived from Antarctic krill. *Wound Repair Regen* 1998;6:50-7.
9. Falabella AF, Carson P, Eaglstein WH, Falanga V. The safety and efficacy of a proteolytic ointment in the treatment of chronic ulcers of the lower extremity. *J Am Acad Dermatol* 1998;39:737-40.
10. Hebda PA, Flynn KJ, Dohar JE. Evaluation of the efficacy of enzymatic debriding agents for removal of necrotic tissue and promotion of healing in porcine skin wounds. *WOUNDS* 1998;10:83-96.
11. Alvarez OM, Fernandez-Obregon A, Rogers RS, et al. Chemical debridement of pressure ulcers: A prospective, randomized, comparative trial of collagenase and papain/urea formulations. *WOUNDS* 2000;12:15-25.
12. Pullen R, Popp R, Volkens P, Fusgen I. Prospective randomized double-blind study of the wound-debriding effects of collagenase and fibrinolysin/deoxyribonuclease in pressure ulcers. *Age and Ageing* 2002;31:126-30.