

The Effects of Active Ingredients of Standard Debriding Agents—Papain and Collagenase—on Digestion of Native and Denatured Collagenous Substrates, Fibrin and Elastin

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Abstract: Debridement of necrotic eschar from wounds can be accomplished with the application of proteolytic enzyme formulations. However, a clear understanding of the biochemical activities of the enzymatic agents is essential for achieving effective wound debridement. This report describes the *in-vitro* evaluation of the active ingredients of several commercial debriding agents. Collagenase and papain/urea were tested in standard enzymatic assays for their ability to digest several substrates found in various types of wound eschars and chronic ulcers. Both showed activity with denatured and, to a lesser extent, native collagenous substrates. Papain/urea effectively digested fibrin but had only slight activity with elastin. Collagenase slightly digested fibrin but was active in digesting elastin. These results indicate that the biochemical activities vary among the active ingredients of topical debriding agents, and this finding should be a consideration in selecting the best treatment for each type of wound.

Supported in part by Rystan Company, Inc., Little Falls, New Jersey

WOUNDS 2001;13(5):190-194

Wound debridement—removal of necrotic or nonviable tissue, or wound eschar, from the wound surface—can be effectively achieved for different types of wounds with the use of proteolytic enzyme formulations. Often, the concern of the wound care provider is choosing which formulation to use. Knowing the biochemical composition of the wound eschar and the biochemical activity of the proteolytic enzyme is very important for making the right choice. Another impor-

tant consideration is to protect healthy, viable tissue around the wound. An ideal debriding agent will effectively and selectively digest only the nonviable tissue within the wound, sparing healthy tissue and leaving a clean wound base that is receptive for wound healing therapies, such as grafts or topical treatments. In many cases, the need for surgical debridement may be avoided entirely.

The purpose of this study was to determine the proteolytic activities of the enzymatic ingredients

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contained in several commercial debriding agents for the digestion of several substrates. The enzymes used were collagenase and papain/urea; for comparisons, trypsin and elastase were used as standard enzymes and fibrinolysin/desoxyribonuclease was used as a fibrinolytic agent. The substrates were selected because they represent major components found in healthy connective tissue or various types of wound eschar. Collagen type I, elastin, fibrin, and porcine skin (a collagenous substrate) were each tested for digestion; additionally, the collagen and porcine skin were heat-denatured to test the ability to digest denatured collagen, as is found in burn wounds and some chronic ulcers.

There have been other reports in the literature of *in-vitro* analyses of enzymatic debriding agents.¹⁻⁶ Carefully designed *in-vitro* testing of enzymatic debriding agents or their active ingredients can provide useful information about the way they can be expected to work in various types of wounds. The results can be used to develop an effective wound care treatment plan and may provide insight into development of improved wound debriding agents.

Materials and Methods

Enzymes contained in standard debriding agents:

- 1:1 mixture of urea USP (reg# 050195) and purified papain (reg# 112295)*
- Collagenase ABC form II, 110,000 BTC units per gram**
- 25 units (loomis) fibrinolysin from bovine blood and 15,000 units deoxyribonuclease from bovine pancreas (reg# N 57317-030-10, 700330)***

Standard enzymes:

- Trypsin—from porcine pancreas (cat# T-8128); Sigma Chemical Co. (Sigma; St. Louis, MO).
- Elastinase—from porcine pancreas (cat# E-1250); Sigma.

Substrates:

- Azocasein (sulfanilamide azocasein) (cat# A-2765); Sigma.
- Collagen (Type I, from bovine Achilles tendon) (cat# C8886); Sigma.
- Elastin-congo red (cat# E-0502); Sigma.
- Fibrin (from human plasma) (cat# F-5386);

Sigma.

- Mediskin II (frozen porcine skin) (cat# MI-104); Brennen Medical Inc., St. Paul, MN.

Biochemical Analysis of Protease Activities

An enzyme preparation from the active ingredients of each debriding agent was assayed for the digestion of the following substrates:

Azocasein hydrolysis.¹ Each enzyme was tested at concentrations ranging from 0.00008 to 4mg/mL in a 50mM Tris HCl buffer (pH 7.4) containing 5mg/mL azocasein to a total volume of 0.5mL. Each mixture was incubated at 37°C for 10 minutes. Next, 0.5mL of chilled 10-percent (weight/volume) trichloroacetic acid (TCA) was added to each sample and incubated on ice for 10 minutes. This mixture was centrifuged at 1500g for four minutes at 4°C. The supernatant from each sample was removed and measured to determine the optical density at 420nm against a blank that did not contain an enzyme or substrate.

Fibrin hydrolysis. The reaction solutions were made by mixing 10mg/mL of human plasma fibrin (Sigma Chemical Co.) in a 100mM N-Tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES) buffer (pH 7.5) containing 0.9 percent NaCl and 0.1mM CaCl₂. Enzymes were tested at a 0.25 and 1mg/mL concentration. These mixtures were placed in a 2cm capped flat bottom glass tube containing a magnetic stir bar and incubated at 37°C. With continuous stirring, samples were removed at 0, 15, 30, 45, and 60-minute intervals and immediately centrifuged. Each supernatant was removed and stored on ice. The ninhydrin test was used to determine the level of free amino acids.⁶

Collagenous substrates digestion. Collagen type I (from bovine Achilles tendon) was mixed with 1M acetic acid, dialyzed against 100mM TES buffer (pH 7.5), and adjusted to final concentration of 7.5mg/mL in 100mM TES buffer containing 37.6mM ethylenediamine tetraacetic acid, 10mM dithiothreitol, 0.1mM CaCl₂ and 0.9 percent NaCl. One aliquot of this collagen solution was denatured in a boiling water bath for seven minutes. Enzymes were tested at 1mg/mL. Each reaction mixture was continuously mixed with a

Table 1. Summary of enzyme activities*

| Enzyme | Substrate | | | | | | |
|----------------------|----------------------|--------|-----------------------|-------------------------------|-----------------|-------------------------|---------|
| | Casein (protein std) | Fibrin | Porcine skin (native) | Porcine skin (heated-denat'd) | Native collagen | Heated-denat'd collagen | Elastin |
| Trypsin (enzyme std) | +++ | +++ | ++ | ++ | + | ++ | - |
| Papain/urea** | ++ | ++ | + | ++ | + | ++ | +/- |
| Fibrinolysin/DNase | - | +/- | - | - | nd# | nd | - |
| Baterial collagenase | +/- | +/- | + | ++ | nd | nd | ++ |

* Relative activity scores were derived from the amino acid equivalents released (mg/ml) by 1mg/ml enzyme after 60 minutes incubation:

- no activity

+/- < 0.5

+ 0.5-1.0

++ 1.0-1.5

+++ > 1.5

** Assayed in papain/urea activating buffer

nd = not determined

magnetic stir bar at 37°C in 2cm capped flat bottom glass tube and samples were removed at 0, 20, 40, 60, 80, and 100-minute intervals and immediately centrifuged. Each supernatant solution was removed and stored on ice. The ninhydrin test was used to measure the level of free amino acids released by enzymatic activity; hydroxyproline levels were also measured.⁶

Both native and denatured porcine skin were used as the collagenous substrate in a two-part experiment. The skin was denatured by boiling Mediskin II in water for two minutes. The skin, both native and denatured, was weighed to yield a 25mg/mL collagen substrate concentration in 100mM TES buffer (pH 7.5) containing 0.9 percent NaCl and 0.1mM CaCl₂. The skin was cut into 2mm x 5mm pieces for better digestion. The enzymes were tested at a 1mg/ml concentration. These mixtures were continuously mixed with a magnetic stir bar in 2cm capped flat bottom glass tubes and samples were removed at 0, 15, 30, 45, and 60-minute intervals and immediately centrifuged. Each supernatant was removed and stored on ice. The ninhydrin test was used to measure the level of free amino acids.⁶

Elastin hydrolysis.² The reaction mixture in this assay consisted of enzymes at a 1mg/mL concentration (except elastinase, which was tested at a 0.11mg/mL concentration) and the elastin

substrate (Elastin-congo red) with a concentration of 6.6mg/mL in 100mM TES buffer (pH 7.5) containing 0.9 percent NaCl and 0.1mM CaCl₂. These mixtures were stirred continuously (as described above). At 0, 30, 60, 90, and 120-minute intervals, a 0.6mL sample was removed, added to 0.4mL 0.7M phosphate buffer (pH 6.0), and immediately centrifuged. The optical density of each supernatant was measured at 495nm.

Results

The enzymes were tested with various substrates under standard conditions. Casein was used as a nonspecific protease substrate. The results are summarized in Table 1. Trypsin was used as a standard protease with broad activity in digesting many proteins. Trypsin showed some activity with each of the substrates except elastin, which, like collagen, is relatively resistant to many proteases. Papain/urea had a similar broad activity with many substrates, except for elastin, with which it showed slight activity. The fibrinolysin/desoxyribonuclease showed no activity with most of the substrates and only slight activity with fibrin, thus its efficiency as a debriding agent is minimal. The collagenase showed the expected activity with native skin (collagenous substrate) but higher activity with heat-denatured substrate; this collagenase formulation also

showed slight activity with casein and fibrin and was significantly active in digesting elastin. The results with collagenase suggest that traces of other enzymes may be present in the formulation.

Further testing was done to determine the selectivity of papain/urea for digesting denatured and sparing native collagen. Trypsin was used as a standard protease and a partially-purified extract from bovine tendon was used as the collagenous substrate. The experiment was designed to measure total protein and collagenous protein solubilized by the enzymes. The solubilized fractions were separated by high-speed centrifugation then acid hydrolyzed and assayed for total amino acids and for hydroxyproline, indicative of total protein and collagen, respectively. The results are shown in Table 2. In this experiment both trypsin and papain/urea digested the extract to some extent, solubilizing some collagen—28 percent for trypsin and 32 percent for papain/urea. Both showed increased activity with denatured collagen—papain/urea showed relatively higher selectivity of denatured collagen—58 percent of the solubilized protein was collagenous. *In vivo*, this selective activity with denatured collagenous substrate may be greater due to the presence of serum and other inhibiting factors within the viable tissue surrounding the native collagen.⁷

Discussion

Papain/urea showed a broad range of activity for the solubilization of matrices and extracellular matrix components, including casein, fibrin, native and denatured collagen, and skin; it did not digest elastin. It was more active and selective in digesting collagen that was heat denatured, as both purified tendon collagen type I and the collagenous connective tissue of skin.

This broad activity as measured in these *in-vitro* experiments may translate into very effective tissue debridement activity *in vivo* for a wide variety of escharified chronic wounds.

Collagenase was active in digesting collagen, both native and heat denatured, but it also digested elastin. It did not digest fibrin. As with papain/urea, the collagenase was more effective in digesting heat-denatured collagen and skin. Its

Table 2. Relative digestion of collagenous and total protein components of bovine tendon collagen (mg protein/mL)+

| Substrate | Native | | Heat-Denatured | |
|-------------|--------|--------------------|----------------|--------------------|
| | total | collagenous (C/T)* | total | collagenous (C/T)* |
| Trypsin | 2.38 | 0.67 (28%) | 10.56 | 4.84 (46%) |
| Papain/urea | 1.83 | 0.58 (32%) | 7.61 | 4.38 (58%) |

+ Solubilized fractions from enzymatic digestion were acid hydrolyzed to release free amino acids. These hydrolysates were analyzed for total (ninhydrin assay) and collagenous (hydroxyproline assay) fractions.

* Values for collagenous digests are based on measured hydroxyproline levels multiplied by 10, to adjust for the relative abundance of this diagnostic amino acid (10%) in the major connective tissue collagens (types I and III). The value in parentheses is collagenous/total protein solubilized for each enzymatic digestion (X 100%).

use as a debriding agent should be determined by the type of eschar present in the wound.

The presence of dried fibrinous exudate, as is found in some chronic venous ulcers, should be effectively removed by papain/urea-based products, since the results above demonstrate higher activity against fibrin than the active components of a long-standing fibrinolytic agent*** no longer on the market.

Heat-denatured collagen is the major component of burn eschar. It should be effectively removed by either papain/urea or collagenase. In this study, both enzymes showed higher levels of activity with heat-denatured versus native collagenous substrate, indicating that the burn eschar should be more rapidly removed than surrounding skin. In animal wound studies conducted in our laboratory, experimental wounds containing denatured collagenous eschar were effectively debrided by papain/urea-based ointments.⁷ Additionally, debridement with papain/urea ointments was achieved without inhibition of subsequent wound healing.

Conclusion

Enzymatic debridement is receiving wider usage with the availability and marketing of sev-

eral commercial preparations. Our results indicate that the biochemical activities vary among the active ingredients of these products, and this finding should be a consideration when deciding which debriding agent to use.

*Panafil[®], Rystan Company, Inc., Little Falls, NJ (now distributed by Healthpoint, Ltd., Fort Worth, TX)

**Collagenase Santyl[®], Advanced Biofactures Corp., Lynbrook, NY (now distributed by Smith & Nephew, Inc., Largo, FL)

***Elastase[®], Parke-Davis, div. Warner-Lambert Co., Morris Plains, NJ

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