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BRIEF COMMUNICATION

Fluorimetric *ex vivo* quantification of protease debriding efficacy on natural substrate

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Abstract

Debridement is the process of removal of necrotic and infected tissue to clean a wound or burn and expedite healing. Proteases such as papain, bromelain, and collagenase that promote debridement by degrading proteins in the dead tissue are in use today. However, the only method to measure debriding efficacy in vitro is the fluorescent monitoring of the digestion of an Artificial Wound Eschar (AWE) substrate. This AWE substrate contains a pellet of only three eschar matrix proteins collagen, elastin, and fibrin which do not account for the complexity and the composition of necrotic tissue. Here, we describe an ex vivo method using dry necrotic full thickness human skin and ortho-phthalaldehyde (OPA), a molecule commonly used for sensitive fluorimetric protein detection to monitor debridement activity. We advocate this simple yet sensitive approach to detect debridement efficacy that can readily be used commercially to benchmark products prior to in vivo testing.

1 | INTRODUCTION

Proteases catalyze the breakdown of polypeptides or proteins by specific cleavage of peptide bonds in their substrates via hydrolysis to preserve tissue integrity.[1,2] Identified proteases are classified into seven groups based on the nature of their catalytic site and optimal pH at which they are active. These include aspartic proteases, cysteine proteases, serine proteases, metalloproteases, threonine proteases, glutamic acid proteases, and asparagine peptidases.[3] Proteases promote wound healing by degrading dead tissue[4] and they have been used as active molecules in dermatological products to treat necrotic wounds or non-healing wounds such as skin ulcers. Proteases promote debridement, defined as the removal of non-viable or necrotic tissue, foreign bodies, and bacteria from acute or chronic wounds to expose the underlying viable tissue.[5,6] Various types of wound debridement techniques are currently available in clinical practice: autolytic, surgical, mechanical, bio-debridement, and enzymatic/chemical debridement.[7,8] Enzymatic debridement agents such as papain, bromelain, ficin, collagenase, and trypsin have been developed as they work together with the endogenous enzymes in the wound to clear the dead tissue.[8-10]

However, currently, enzymatic digestion of proteins is monitored either in-gel or in a solution using mostly serine or cysteine proteases and further analysis to determine the protein concentration. Therefore, it is desirable to objectively monitor the efficacy of enzymatic debridement in whole tissue such as burned or necrotic skin in vitro. Currently, the standard model to quantify debriding efficacy in vitro for assessing protease activity/protein digestion is the artificial wound Eschar (AWE) substrate developed by Shi et al.[11] This AWE comprises of a pellet of three wound related extracellular matrix proteins, typically collagen, elastin, and fibrin, each of them tagged with a different fluorophore. The gradual degradation of this matrix can be measured by progressive increase in fluorescence intensity in the bottom chamber of a Franz diffusion cell.[11] However, this model utilizes only

Abbreviations: AWE, Artificial Wound Eschar; OPA, Ortho-phthalaldehyde.

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artificial proteins and cannot account for the complexity and composition of necrotic tissue (ie, other proteins, lipids, saccharides, blood vessels, coagulation products, bacteria). Therefore, it would be desirable for the scientific community and debriding product industry to have a method available to accurately measure proteolytic digestion in a realistic skin sample that contains all the aforementioned constituents.

We propose a new method based on ortho-phthalaldehyde (OPA), a molecule commonly used in sensitive fluorimetric protein detection such as residual protein on surgical devices, biofilm quantification, and gas chromatographic peptide labeling[12] and also for total protein quantification in solution form.[13] OPA is not fluorescent in its native form, but once activated by reduced sulfhydryl groups it specifically reacts with primary amines in proteins to produce a fluorescent indole derivative, thus resulting in high sensitivity with low background (ng-level limits of detection for amino acids) (Figure 1A).[13] Primary amino groups are only found at the N-terminus of proteins and at the "ε" position of lysines. As a primary amino group is produced after every proteolytic cleavage event, the concentration of primary amino groups is an adequate proxy for the extent of sample digestion, more so than the total protein concentration, which would also include dissolved, undigested macromolecules and does not increase with proteolysis. In the present communication, we describe the application of OPA in the assessment of the debriding efficacy of proteolytic enzymes.

In our study, a dry necrotic full thickness human skin is placed in contact with the debriding proteolytic solution and the digested peptides are separated by centrifugation at 24 hours. The advantage of this technique over the previous analytical protocol[11] is the fact that it uses natural skin and it measures overall protein digestion instead of only three proteins (fibrin, collagen, and elastin) as in the AWE.

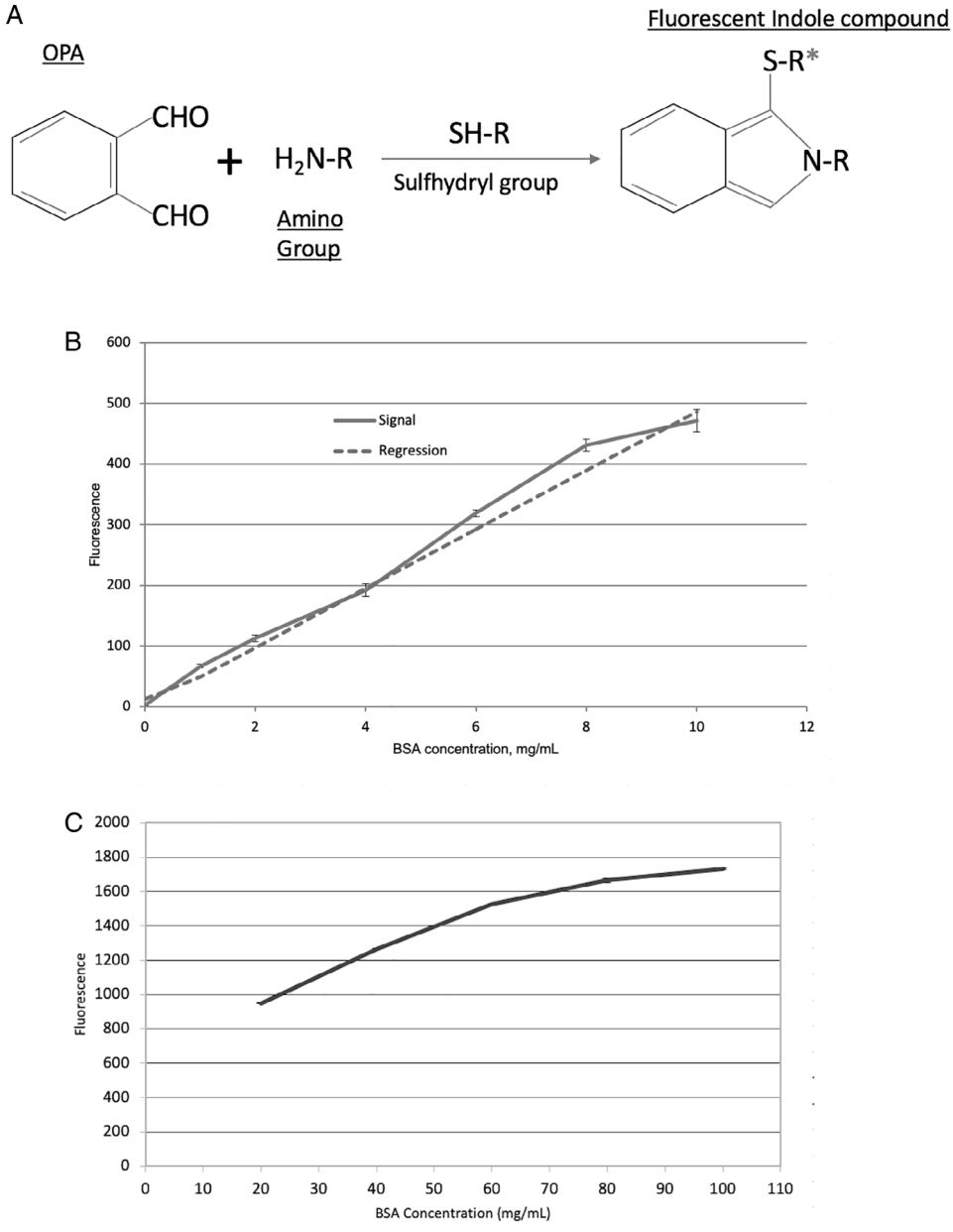

FIGURE 1 A, Reaction of OPA and primary amino groups in the presence of sulfhydryl groups yields a fluorophore which can be detected Fluorimetrically. B,C, Linearity response of the assay over 1-10 mg/mL (B) and, over 20-100 mg/mL protein concentration (C)

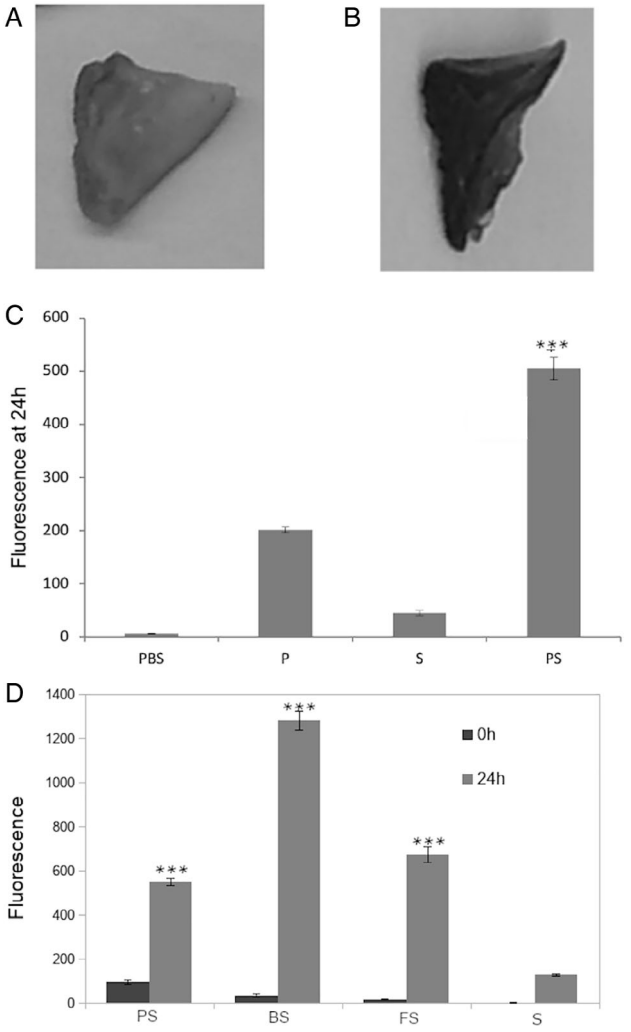

FIGURE 2 A,B, human skin before (A) and (B) after 72 hours in a warm room to generate dried full thickness necrotic skin. C, Fluorescence monitoring by OPA assay of the proteolysis of eschar by papain showing a greater increase vs eschar and papain alone. PBS, phosphate buffered saline; P, papain; S, eschar without papain; PS, Eschar with papain. D, comparison of the debriding efficacy at 24 hours of three proteolytic enzymes using the OPA assay: (papain (PS), bromelain (BS) and ficin (FS)) on eschar showing significantly greater digestion vs control eschar in PBS (S). Results are represented as the mean ± SD for n = 3 experiments, each measured three times. Statistical analysis was carried out using the Student *t*-test (***$P < .001$)

Moreover, it does not require a complex Franz cell chamber and the results are not skewed by the product diffusion rate through a filter membrane as in the Franz cell chamber.

2 | MATERIALS AND METHODS

2.1 | Materials

Healthy skin samples from abdominal reduction surgery were obtained from the Royal London Hospital. The skin was cut into small pieces about 100 mg in weight and incubated at 37°C in a hot air room for 3 days to generate dry full thickness necrotic skin (Figure 2A,B). OPA, papain, stem bromelain, ficin, and β-mercaptoethanol were obtained from Sigma. The plate reader used was a Synergy HT microplate reader (cat. 12 926 527, Bio-Tek Instruments, Thermo Fisher Scientific, UK).

2.2 | Methods

Linearity of the test response to protein concentration was carried out using BSA: 20 μL BSA solutions (1-10 or 10-100 mg/mL in PBS) were dispensed in a 96-well plate, added with 200 μL of an OPA solution (1 mg/mL PBS, supplemented with 0.2% β-mercaptoethanol), and, following 5 minute incubation, fluorescence was read (excitation at 360 nm, emission at 480 nm). The 5-minute time period was chosen as the point when the reaction was kinetically complete as measured in a separate kinetic run (data not shown).

2.3 | Debridement assay

The skin samples were rinsed with PBS until the OPA reading was insignificantly different than that of PBS to remove any material on the surface of the skin that may increase the background readings.

For each test, four solutions were prepared as follows:

PBS: PBS alone (Negative control).

P: Papain 2 mg/mL in PBS (No substrate control).

S: Dry full thickness necrotic skin in PBS (No enzyme control).

PS: Papain 2 mg/mL in PBS plus dry full thickness necrotic skin (Positive).

For experiments involving skin (S and PS), the volume of the PBS or debriding solution was 20 times the weight of the eschar; this allowed us to take into account slight differences in the weight of the skin. The vials were incubated at 37°C for 24 hours. At the end of this period, 20 μL of each sample were transferred in triplicate onto a 96 well plate and added with 200 μL of the OPA solution. Fluorescence was read after 5 minutes incubation. The subsequent debridement assay involving the other proteases followed the same setup with each enzyme substituted for papain.

3 | RESULTS

Results of the linearity experiments (Figure 1B,C) confirmed the linearity of the assay over the 1 to 10 mg/mL range. Above 20 mg/mL (1000 fluorescence units) the response was no longer linear indicating the need to dilute samples exceeding this reading.

The results of the debridement assay (Figure 2C) show a significant increase in fluorescence in the enzyme plus dry full thickness necrotic skin sample, this being greater than the sum of that of skin and enzyme alone, indicating the generation of additional free amino groups, a consequence of skin digestion. We then compared the

debriding efficacy of three proteolytic enzymes: papain, bromelain, and ficin using the same experimental conditions. Figure 2D shows significantly greater debridement compared to control, with bromelain approximately twice as potent at debriding as papain and ficin.

4 | DISCUSSION

Wound debridement is a medical procedure aimed at facilitating the healing of chronic wounds and preventing their infection. Although it is normally carried out surgically, there is a demand for non-surgical alternatives such as enzymatic debridement because surgical debridement requires specialized medical personnel and is associated with risks of bleeding and damage to the surrounding healthy tissues and vital structures such as nerves.[14] Enzymatic debridement is also used in combination with other techniques such as surgical debridement for better treatment of chronic wounds.[15]

Some products, based on papain, bromelain, and collagenase, have been developed and marketed for this purpose. In this respect, it would be desirable for the research community to have available a tool to benchmark the efficacy of debriding formulations so as to optimize their composition before progressing toward expensive in vivo trials. To our knowledge, the only one such assay is the AWE protocol developed by Smith and Nephew[11] which, however, relies on an artificial eschar model not representative of actual tissue complexity and relying on an apparatus (the Franz diffusion cell) which further complicates readouts and introduces perturbations such as diffusion. In this work, we propose a completely novel method directly applicable to human tissue samples, which is easy to set up, fast, and inexpensive. The aim of the experiments presented in this report was to validate a new assay for enzymatic debridement that was closer to human tissue than the artificial eschar and we did not investigate much shorter clinically relevant time points, such as those prescribed for commercial enzymatic debriding formulations. Moreover, by exploiting the fluorescent derivatization of primary amino groups, only generated during proteolytic hydrolysis of the peptide bond, it achieves a higher level of sensitivity and specificity than total protein assays and can be used by researchers to characterize debriding formulations in a more realistic setting including against wet necrotic eschar involving biofilm and burn tissue.

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CONFLICT OF INTEREST

We declare no conflicts of interest.

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