Specific protease activity indicates the degree of *Pseudomonas aeruginosa* infection in chronic infected wounds

Dirk Wildeboer[1,#], Katja E. Hill[2], Fiona Jeganathan[1], David W. Williams[2], Andrew D. Riddell[2,3], Patricia E. Price[3*], David W. Thomas[2], Phil Stephens[2], Ramadan A. Abuknesha[1] and Robert G. Price[1]

1) King's College London, Pharmaceutical Science Division, Analytical Sciences Research Group, School of Biomedical & Health Sciences, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK
2) Cardiff University, School of Dentistry, Tissue Engineering & Reparative Dentistry, Heath Park, Cardiff CF14 4XY, UK
3) Cardiff University, School of Medicine, Department of Wound Healing, Heath Park, Cardiff CF14 4XN, UK.

[#] Corresponding author and reprint requests: Dr Dirk Wildeboer, Middlesex University, Natural Science Department, School of Health and Social Science, The Burroughs, London NW4 4BT, UK.
Email: d.wildeboer@mdx.ac.uk
Phone: 0044 (0)208 411 5537
Fax: 0044 (0)208 411 6774

* Present address: Prof. P. Price, Cardiff University, School of Healthcare Studies, Ty Dewi Sant, Heath Park, Cardiff, CF14 4XN

Abstract

Chronic non-healing wounds are a major health problem with resident bacteria strongly implicated in their impaired healing. A rapid-screen to provide detailed knowledge of wound bacterial populations would therefore be of value and help prevent unnecessary and indiscriminate use of antibiotics; a process associated with promoting antibiotic resistance. We analysed chronic wound fluid samples, which had been assessed for microbial content, using 20 different fluorimetric peptide substrates to determine whether protease activity correlated with the bacterial load. Eight of the peptide substrates showed significant release of fluorescence after reaction with some of the wound samples. Comparison of wound fluid protease activities with the microbiological data indicated that there was no correlation between bacterial counts and enzyme activity for most of the substrates tested. However, two of the peptide substrates produced a signal corresponding with the microbial data revealing a strong positive correlation with *Pseudomonas aeruginosa* numbers. This demonstrated that short fluorescent peptides can be used to detect protease activity in chronic wound fluid samples. The finding that two peptides were specific indicators for the presence of *P. aeruginosa* may be the basis for a diagnostic test to determine wound colonisation by this organism.

Keywords: chronic venous leg ulcer; AMC-peptides; *Staphylococcus aureus; Pseudomonas aeruginosa;* bacterial protease; chronic infection; skin wounds

Introduction

Chronic non-healing skin wounds are a major world health problem; occurring in three main forms, pressure ulcers, venous leg ulcers and diabetic foot ulcers. These wounds represent an unrecognised cause of disability and distress in the aged population [1] and, importantly, are estimated to consume 2–4% of the total healthcare budget in European Union countries [2, 3]. The incidence of these wounds is rising inexorably with the increased age of the population and corresponding increases in obesity and type II diabetes. These additional comorbidities often result in lower limb amputations and sepsis as frequent chronic wound complications. Uncertainty about the effectiveness of the various wound dressings available [4] highlights the ambiguities involved in the treatment of venous ulcers.

Whilst the aetiology of chronic wounds is multi-factorial, bacteria play an important direct and indirect role in the chronicity of the disease [5]. Through production of proteases and other metabolites, bacteria may both modulate responses in the resident cellular populations and directly degrade extracellular matrix. Additionally, bacteria also stimulate innate and adaptive inflammatory responses in the dermis with the generation of oxidative stress in the wound environment [6]. Wounds support a diverse microflora [7]. The importance of anaerobic organisms in perpetuating wounds has been observed both *in vivo* [5, 8] and *in vitro* [6], although relatively few detailed microbiological studies have been undertaken [9]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most frequently isolated aerobic species from these wounds [5, 9]; both species being opportunistic pathogens commonly found colonising healthy skin [10]. *Staphylococcus aureus* can be highly pathogenic when invading the skin barrier [11], and impairs wound-healing via the expression of a wide range of virulence factors. In the wound environment, the formation of *P. aeruginosa* biofilms results in significantly larger ulcers and delayed wound-healing [10] and contributes to antibiotic resistance, particularly ciprofloxacin [12].

Rapid analysis of bacteria populating particular wounds would be extremely useful in clinical practice, avoiding unnecessary and arbitrary use of antibiotics, with its known promotion of antimicrobial resistance [13]. Unfortunately, current microbiological analysis of wound fluid samples takes 48-72 h for aerobic species and over 7 days for slow-growing strictly anaerobic bacteria.

Consequently, antibiotic prescribing for these patients is largely empirical with the over-prescription of antibiotics [13] and antimicrobial resistance being a common feature in these wounds [9].

Bacterial enzymes released into the local environment can be measured using well established analytical processes based on chromogenic or fluorogenic substrate assays. Proteases have great potential as specific markers of infection. Novel approaches to infection treatment based on proteases have been extensively researched [14, 15] with the emphasis being on reducing protease activity in the wound using absorbent dressings [16, 17] or protease inhibitors [18, 19]. Bacterial proteases released into the wound environment have a variety of effects on defence and healing mechanisms. These include the activation of matrix metalloproteinases (MMPs) by proteolytic removal of the inhibitory pro-domains [20], targeting the fibrinolytic system [21] and affecting macrophage activity [22]. Bacterially secreted endoproteases, quantified using fluorescent peptide substrates, can be used for direct identification of specific pathogenic bacteria [23]. Wildeboer *et al.* [23] showed that protease activity, analysed using short peptide libraries, was most sensitive and specific for the detection and quantification of *P. aeruginosa*. Hence, characterising protease activity in wound fluid samples, with substrates that specifically detect bacterial proteases, holds potential for a rapid diagnosis.

In the present study, the objective was to identify protease activity against specific peptide substrates that correlated with the bacterial load of wound fluid samples from patients with chronic infected wounds, particularly *P. aeruginosa.*

Materials and Methods

Chemicals and reagents

Chemicals and reagents, if not specified otherwise, were obtained from Sigma-Aldrich (Poole, UK). Peptides were purchased from Sigma-Aldrich, Bachem (St. Helens, UK), Biomol (Exeter, UK) and Calbiochem (Nottingham, UK). Twenty different peptides labeled with a carboxy-terminal 7-amino-4-methylcoumarin (AMC) were included in this study (Table 1).

Patients

Following local research ethics committee approval and after obtaining patient informed written consent, patients with chronic venous leg ulceration attending the Wound Healing Research Unit, University Hospital of Wales, Cardiff, UK were recruited to provide swab and wound fluid samples. Patients were selected that had highly exudating wounds and included four male and six female patients (Table 2). The participant's ages ranged from 62 to 88 years, with a mean age of 74.1 ± 9.2 years and wound duration ranging from 10 months to 27 years. The wounds were all located on the lower legs, with one a malleolus wound, four semi-circumferential and five circumferential wounds. Ulcer causation was venous disease in five cases, one burn, one trauma and four of unknown aetiology. The patients were receiving a range of medication. Three patients with infected wounds, assessed by experienced wound healing experts, were taking oral antibiotics at the time of wound fluid sampling. Also of note, participant 2 was also using potassium permanganate soaks, a topical treatment effective against *Pseudomonas*.

Wound fluid samples

Wound fluid from patients was collected using a totally non-invasive method by extracting the fluid from wound dressings [24]. At the same time, a microbiological sample of the wound surface was obtained using Amies charcoal transport swabs (Sterilin, Newport, UK). Samples were transported to the microbiology laboratory where the wound fluid was eluted no later than 30 min after removal of the dressing from the wound. The dressings were cut into 5 cm x 5 cm squares placed in a sterile Petri-dish with 12.5 mL of wound fluid elution buffer (0.1 M Tris-HCl, pH 7.4, 0.1% Triton X-100) and agitated on a tilt board at 4°C for 4 h. The fluid was squeezed out of each dressing using sterile forceps and the eluate of each dressing recombined in a sterile bottle. A 100 µL portion of this fluid was removed for microbial analysis. Wound fluid samples were aliquoted and stored at -80°C in a locked Human Tissue Authority approved freezer.

Microbiological analysis of wound fluid samples

Microbial swab samples were streaked onto non-selective media plates to assess for the presence of bacteria in the wounds. Wound fluid samples were

extracted from dressings as described above, serially diluted in phosphate buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, *p*H 7.4, PBS) and plated on to selective and non-selective media. *Pseudomonas* agar (PA, LabM, Bury, UK) supplemented with 200 mg/L cetrimide and 15 mg/L nalidixic acid was used for detection of *P. aeruginosa*, and colony identification confirmed by colony morphology, Gram-stain, oxidase and catalase tests as well as PCR. Mannitol salt agar (MSA, Oxoid, Basingstoke, UK) was used for the detection of *S. aureus*. *Staphylococcus aureus* grow as yellow colonies on MSA and identity was further confirmed by Gram-stain and coagulase tests. Blood agar (BA, LabM, Bury, UK) was used for non-selective culture of the whole microflora to obtain a total microbial count for each wound fluid sample.

Protein quantification in wound fluid samples

The total protein concentration of the wound fluid samples was quantified using a BCA kit (Novagen–Merck, Darmstadt, Germany) following the standard protocol for the micro-scale assay. The chronic wound fluid samples were diluted 1:5 and 1:20 in Tris-buffered saline (137 mM NaCl, 10 mM Tris-HCl, *p*H 7.3, TBS) prior to assaying. Absorbance was measured at 584 nm in a micro-plate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) and analysed with the accompanying MARS software using a simultaneously recorded bovine serum albumin reference curve.

Fluorescent assay of protease activity with peptide-AMC substrates

Peptide-AMC substrates were dissolved in DMSO at 5 mg/ml and diluted to 50 µM in PBS. Wound fluid samples were prepared as described above and diluted prior to the assay with an equal volume of Tris buffered saline (137 mM NaCl, 10 mM Tris, pH 7.3, TBS). Aliquots of 10 µl of 50 µM peptide-AMC were pipetted into the wells of a black 96 well micro-titre plate (Greiner, Stonehouse, UK) and 90 µl of wound fluid sample added. The final concentration of substrates in the reaction mix was 5 µM. Fluorescence (excitation wavelength 355 nm, emission wavelength 450 nm) was measured at intervals from the start of the reaction for 12 h following the addition of the wound fluid in a FLUOstar OPTIMA plate reader. Measurements were taken at 5 min intervals for the first

1 h and then every 30 min. Results were corrected for the background fluorescence of the wound fluid sample, as well as for the peptide-AMC.

Results

Microbial status of the wound fluid samples

Pseudomonas aeruginosa was found to be present in all samples bar one, with limited growth seen in sample 1 (Table 2). Coagulase negative staphylococci (CNS) were found in six of the samples and *S. aureus* in five, with samples 3 and 4 containing both organisms. Other organisms detected included *Proteus* and *Corynebacterium* spp.

Total protein content of chronic wound fluid samples

The total protein concentration in the wound fluid samples ranged between 873 μg/mL and 510 μg/mL, with a mean of 697 μg/mL and a median of 719 μg/mL (Table 2). The total protein concentration did not correlate with any of the quantitative bacterial counts or protease activity detected with the fluorescent peptides.

Specific protease activity in wound fluid samples

Aliquots from ten chronic wound fluid samples (Table 2) were assayed with each of the peptide-AMC substrates (Table 1) to determine protease activity. Reaction and measurement conditions were optimised for maximum sensitivity and a rapid, but reliable response. Eight substrates out of the 20 tested showed a significant increase in fluorescence over the 12 h reaction time with all or some of the ten wound fluid samples. The slope of the initial linear phase was used to calculate enzyme activity against these eight substrates, as shown for samples numbers three and six (Fig. 1). Figure 2 shows a single point analysis of the fluorescence intensity after 1 h reaction time for all 10 wound fluid samples with the eight substrates showing the strongest response.

Analysis of protease activities obtained using the peptide-AMC substrates for a relationship with the microbiological data showed a positive correlation for initial enzyme activity, and for 60 min and 6 h signal intensity, with the microbial counts for *P. aeruginosa*. Scatter plots of all the data indicated that there was

no correlation between bacterial count and enzyme activity for most of the substrates. However, Pearson correlation analysis confirmed that enzyme activity measured with two of the 20 peptide-AMC substrates tested (VLK and AFK) were positively correlated with the *P. aeruginosa* quantitative microbial counts from the ten wound fluid samples ($p<0.01$, $r>0.8$, Table 3). There was only limited evidence for a moderate relationship between enzyme activity and *S. aureus* counts, and insufficient evidence for a relationship with total bacterial numbers (Table 3). Regression analysis of the 6 h data, using the AFK substrate with the ten wound fluid samples, revealed that enzyme activity against this substrate was a sufficient predictor of the number of *P. aeruginosa* found in the wound fluid sample (R^2 = 72%, Fig. 3A) but not for *S. aureus* (R^2 < 35%, Fig. 3B) or total number of bacteria (Fig. 3C).

Discussion

Bacterial proteases are a promising target for the analysis of infected wounds [25, 26] with the ultimate aim being to improve patient treatment regimes and overcome the limitations of currently employed antimicrobial therapies; often prescribed empirically. Previous work, using purified bacterial proteases and cultivated clinical isolates of *P. aeruginosa*, has shown that assays with specific peptide substrates can be used to rapidly quantify bacterial pathogens in a given sample [23]. Furthermore, recent identification and characterisation of proteases specific for pathogenic organisms [27, 28] holds possibilities for the development of more specific novel diagnostic approaches as a possible addition to currently employed microbiological and molecular methods. To achieve this goal, assays are required, which allow testing without the need for lengthy purification steps of the patient sample, and which then produce robust and rapid quantitative results.

The data presented showed that specific peptide substrates could be used to quantify protease activity in *ex vivo* samples from chronic infected wounds. We also demonstrated that rapid analysis could be directly achieved with these patient wound samples. However, only two of the wide range of peptide substrates screened, were identified as having efficacy as specific indicators for the presence of *P. aeruginosa* in wounds. None proved appropriate for detection of *S. aureus*.

Bacterial proteases play an important role in pathogenicity [29] and *Pseudomonas* proteases have been shown to play a significant role in keratitis, affecting both host defence and healing mechanisms [30]. Hence, profiling of protease activity in a wound fluid sample could provide important information on wound healing, particularly if wound proteases were measured over time. Furthermore, the identification of species-specific substrates could be the first step to developing specific inhibitory molecules that might limit the pathogenicity of an infecting organism, not only by improving the host's ability to overcome infection but also by promoting wound healing.

A number of the patients included in this study had received various forms of treatment with bactericidal agents. However, five of the ten samples showed *P. aeruginosa* counts of > 1 x 10^6 cfu/mL*;* with the extracted wound fluid of only one patient being negative for this organism. This implies that *P. aeruginosa* remained largely unaffected by the antimicrobial treatment regimens being used; highlighting the importance of specifically quantifying and targeting this organism. Previous studies have shown that *P. aeruginosa* is often under represented by microbial culture, being present in unculturable form [5]. Formation of biofilms by *P. aeruginosa* in infected wounds delayed healing in a diabetic mouse model [31], demonstrating the importance of detecting and targeting this organism in wound healing therapy.

Statistical analysis revealed a significant correlation for only two of the 20 substrates tested. The identified substrate, Suc-AFK-AMC, had previously been shown to be a good marker for *P. aeruginosa* protease activity using cultured reference strains [23]. The second positive substrate, Boc-VLK-AMC, also cleaved the carboxy-terminal of a lysine residue, indicating that this may be a key element in its substrate specificity. Further studies are needed to identify the importance of the remaining peptide amino acids present and to explore the possibility of increasing substrate-specificity by modification of these. Test conditions in this study were optimised in such a way that significant results could be obtained within a 1 h reaction time. Further testing of a larger number of patient samples will be required to confirm these results, and to obtain further insight into the effect that previous patient antimicrobial treatments may have on test outcomes. Our results demonstrate that a rapid test, based on specific enzyme activity, holds exciting potential for identifying pathogenic organisms

directly from patient samples such as wound fluid, without the need for time-consuming processing or purification. Such systems could ultimately become useful prognostic indicators of non-healing in the wound healing clinic and hence a significant aid to patient treatment.

Acknowledgements

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Tables

Table 1. Amino acid sequences of the 20 peptide-AMC substrates included in this study. Peptide amino acid sequences read from amino to the carboxy terminus, with all having a 7-amino-4-methylcoumaryl group (AMC) at the carboxy-terminus. Some of the peptides also carry a protective group at the α-amino group of their first amino acid.

No	Peptide substrate abbreviation
1	AAF-AMC
2	*Suc*-AAF-AMC[a]
3	*Z*-GGL-AMC[b]
4	*Z*-LLE-AMC[b]
5	*Suc*-AFK-AMC[a]
6	*Boc*-QAR-AMC[c]
7	*Boc*-VPR-AMC[c]
8	PFR-AMC
9	*Suc*-LLVY-AMC[a]
10	*Z*-RLRGG-AMC[b]
11	*Z*-AAN-AMC[b]
12	*Z*-GAM-AMC[b]
13	*Z*-GAH-AMC[b]
14	*Suc*-IIW-AMC[a]
15	*Boc*-VLK-AMC[c]
16	*Z*-GGR-AMC[b]
17	*Boc*-GKR-AMC[c]
18	*MeOSuc*-AAPV-AMC[d]
19	*Ac*-DEVD-AMC[e]
20	*Suc*-GPLGP-AMC[a]

[a] *Suc*, succinyl
[b] *Z*, carboxy benzoyl
[c] *Boc*, tert. butyl-oxycarbonyl
[d] *MeOSuc*, methoxy-succinyl
[e] *Ac*, acetyl

Table 2. Summary of key patient data, *P. aeruginosa* quantification and total protein concentration in chronic wound fluid samples. Concentration values displayed are the mean of two independent measurements, each carried out in triplicate.

Patient no.	Age (years)	Sex	Wound duration (months)	*Pseudomonas* sp.[a] (cfu/mL)[b]	Total protein (µg/mL)
1	88	M	36	limited growth	873
2	65	F	13	6.3×10^6	783
3	84	M	216	6.5×10^4	827
4	63	M	18	3.4×10^9	717
5	68	F	216	4.5×10^6	721
6	79	F	180	4.2×10^6	563
7	80	F	10	n.d.[c]	562
8	62	F	48	4.8×10^5	692
9	74	F	17	1.6×10^4	720
10	78	M	138	7.8×10^6	510

[a] counts derived from *Pseudomonas* agar plates
[b] cfu, colony forming units
[c] not detected

Table 3: Pearson correlation of protease activity in chronic wound fluid samples using two peptide-AMC substrates (VLK and AFK). Enzyme activity was measured as initial activity over 1 h and by single point analyses after 60 min and 6 h. Bacterial counts for *P. aeruginosa*, *Staphylococcus* spp. and CNS) and total bacteria, obtained from chronic wound swab sample cultures on selective (PA and MSA) and BA are also shown.

Peptidase activity with peptide-AMC		Pearson correlation	*P. aeruginosa*	*Staphylococcus* spp.	Total bacterial counts
VLK	initial rate	r[a]	0.840	0.683	0.526
		p[b]	0.009	0.043	0.118
	60 min	r[a]	0.864	0.675	0.524
		p[b]	0.006	0.046	0.120
	6 h	r[a]	0.860	0.634	0.561
		p[b]	0.006	0.066	0.091
AFK	initial rate	r[a]	0.794	0.658	0.514
		p[b]	0.019	0.054	0.128
	60 min	r[a]	0.869	0.704	0.558
		p[b]	0.005	0.034	0.094
	6 h	r[a]	0.919	0.820	0.669
		p[b]	0.001	0.007	0.035

[a]r, Pearson correlation coefficient
[b]p, probability value

Figures

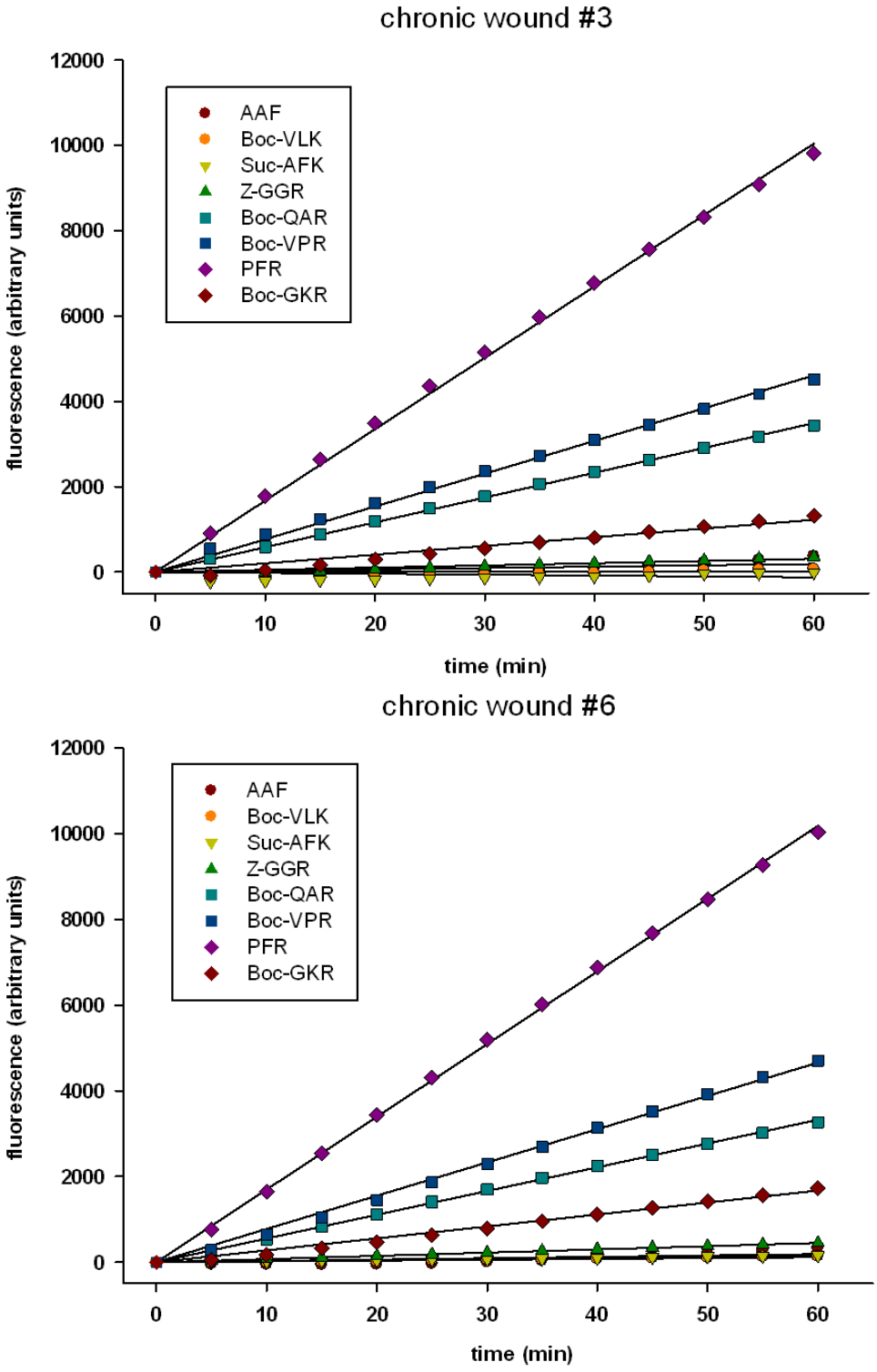


Fig. 1. Initial protease activity plots for two selected wound fluid samples with the eight peptide-AMC substrates that showed the strongest response (A – patient sample 3, B – patient sample 6)

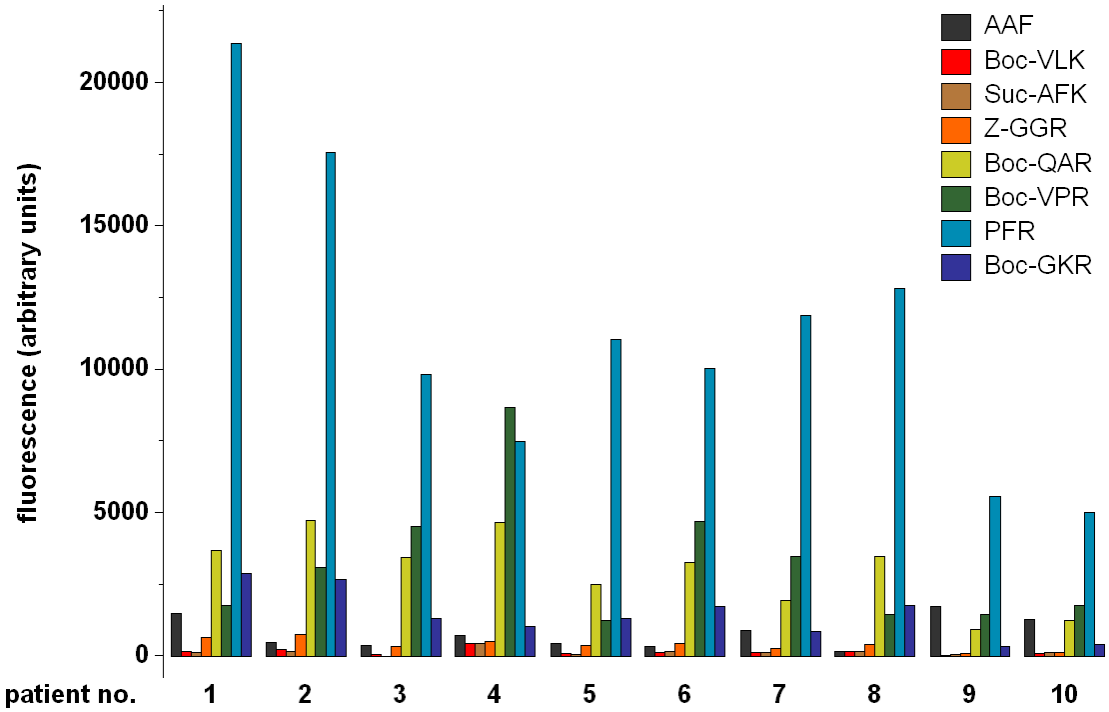
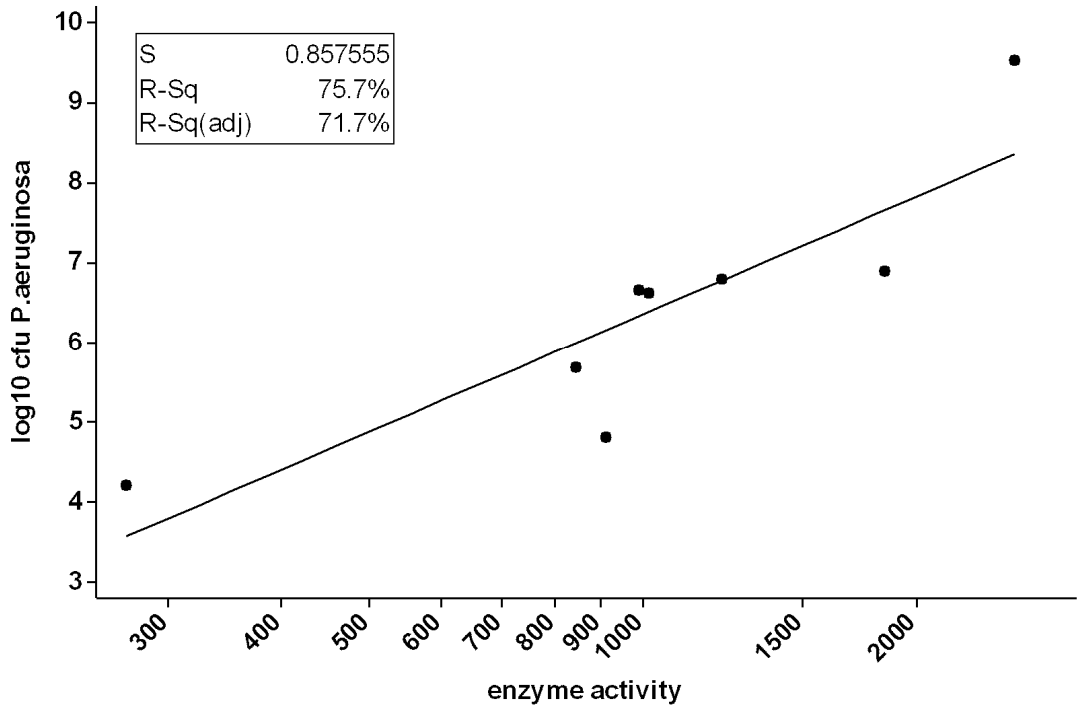
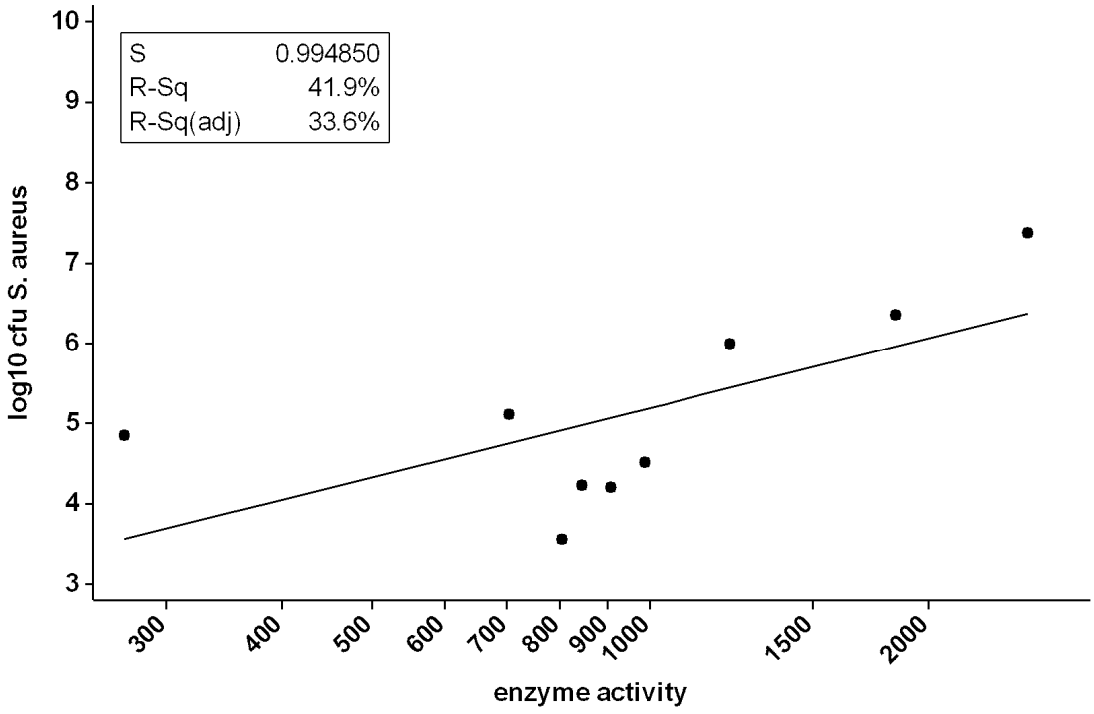


Fig. 2. Fluorescence intensity after 60 min reaction time of the wound fluid samples with the eight peptide-AMC substrates that showed the strongest response


S 0.857555
R-Sq 75.7%
R-Sq(adj) 71.7%
log10 cfu P.aeruginosa
enzyme activity
10
9
8
7
6
5
4
3
300
400
500
600
700
800
900
1000
1500
2000

1


S 0.994850
R-Sq 41.9%
R-Sq(adj) 33.6%
log10 cfu S. aureus
enzyme activity
10
9
8
7
6
5
4
3
300
400
500
600
700
800
900
1000
1500
2000

2

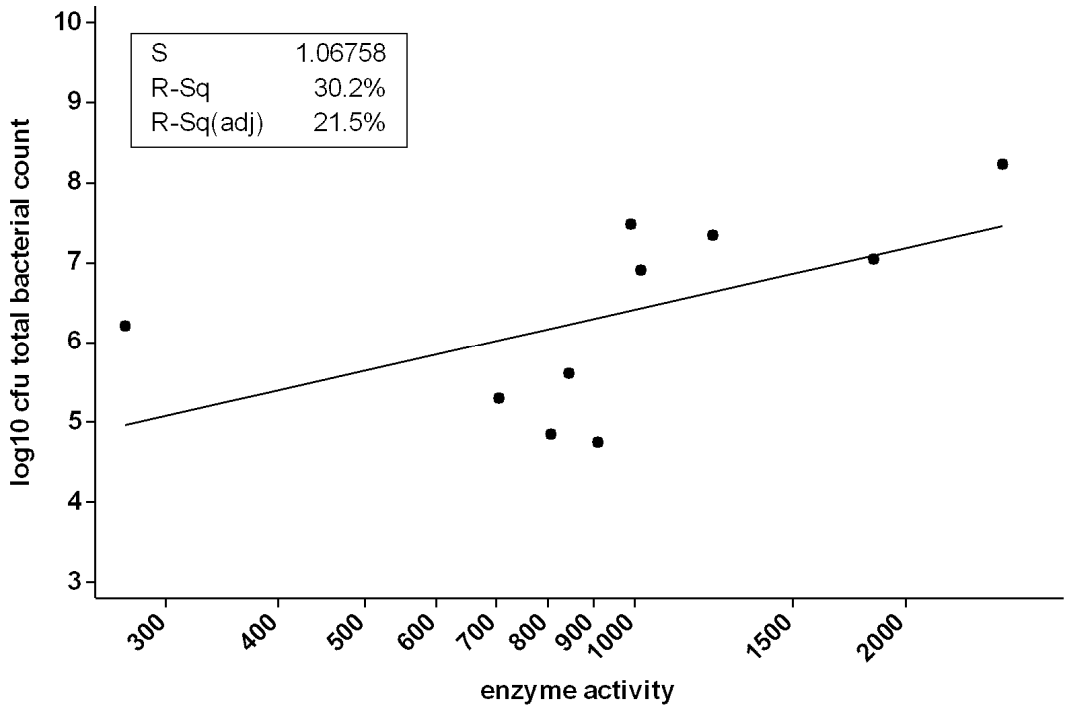


Fig. 3. Regression analysis of quantitative bacterial counts for *P. aeruginosa* (A), *S. aureus* (B) and total bacterial count (C) with 6 h enzyme activity measured with Suc-AFK-AMC substrate