Expression of a cGMP Compatible *Lucilia sericata* Insect Serine Proteinase Debridement Enzyme

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Previously, we demonstrated the effectiveness of a research grade recombinant chymotrypsin,[1] derived from the larvae of Lucilia sericata, in "debriding" slough/eschar from venous leg ulcers ex vivo. Furthermore, we were able to formulate this enzyme for successful delivery to in vitro wound healing assays, from a prototype hydrogel wound dressing,[2] and showed that enzyme delivered in this way could degrade wound tissue ex vivo.[3] Recently, to progress biotechnological development of the enzyme as a potential therapeutic product, we explored expression using current good manufacturing practice (cGMP) guidelines, and now report that a recombinant chymotrypsin I zymogen from L. sericata can be expressed in the cGMP acceptable strain of Escherichia coli (BLR–DE3). In addition, the conditions required for purification, refolding and activation of the chymotrypsinogen have been determined. The activated enzyme was stable, and effective in digesting wound slough/eschar tissue. To summarise, we have successfully initiated the production and characterisation of a novel cGMP compatible product for use in future clinical trials. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000: 000–000, 2012
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Introduction

The treatment of wounds such as pressure ulcers, venous leg ulcers, and diabetic foot ulcers is estimated to cost the UK's National Health Service ~£1 billion per year,[4] a cost, which looks set to rise, as it has been suggested that the number of poorly healing surgical wounds may increase in the future. Factors contributing to this rise in costs include the growing complexity of surgery, an increase in hospital-acquired infections, and increased longevity. Furthermore, the necrotic tissue present in poorly healing wounds is considered to promote bacterial growth, to inhibit the penetration of antibiotics, and to prevent the formation of granulation tissue, which would support subsequent re-epithelialisation.[5,6] Therefore, many clinicians view the removal of slough and eschar from wounds, a procedure termed debridement, as an essential clinical step in wound management.[7] Debridement may be accomplished surgically, or through the use of topical agents containing enzymes such as bacterial collagenase (Santyl®).

Biosurgery, using larvae from the greenbottle fly *Lucilia sericata*, is considered to be an effective method of wound debridement.[8–10] As a necessary step toward understanding the biochemical mechanisms responsible for the effective debridement seen during biosurgery, we investigated the enzymatic activity present within maggot secretions and/or excretions and detected in wounds.[11] We discovered aspartyl, metallo- and serine proteinase activities[12] with the latter shown to degrade a variety of extracellular matrix components, promoting migration of fibroblasts in the process.[13–15]

As a result, we then began to develop maggot enzymes as wound management agents, beginning with a chymotrypsin, for debridement. Initially, the enzyme was expressed to research grade in insect cells.[1] Further to this, preliminary analyses of yeast, mammalian, and bacterial expression systems (see supplementary data I) resulted in the selection of *Escherichia coli* as the system of choice for the production of a good manufacturing practice (cGMP) grade material.

Here, we report further on the progress of this biotechnology; the expression of maggot chymotrypsinogen I in bacteria, and the purification, refolding and molecular characterization of the resulting recombinant protein as an activated insect serine protease (ISP). We can also confirm the potential of this enzyme as a wound debridement agent.

Material and Methods

Cloning and expression of ISP cDNA

In feasibility studies, attempts were made to express the chymotrypsinogen c DNA using a number of cGMP acceptable systems. Expression in yeast failed, and yields from HEK and CHO were very low (see author supplementary data I). Herein, we report on the cloning and expression of

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Figure 1. The amino acid sequence (a) of recombinant *Lucilia sericata* chymotrypsinogen I (ISP), and its appearance post-purification and refolding on SDS-PAGE (b). The predicted mass of this polypeptide is 25,456.8 Da.

Activation by immobilised trypsin removes the MFEGR peptide. The bold underlined black residues then combine to form the ISP active site. A second potential endoproteolytic cleavage site is indicated between residues 109/110 (LI), and a 12% SDS-PAGE gel of the recombinant ISP run under reduced (lane 3) and non-reduced conditions (lane 2) is shown in Figure 1b. Lane 1 contains molecular weight markers.

an untagged chymotrypsinogen 1 in *E. coli*. A cDNA encoding the chymotrypsinogen I from the maggot *L. sericata*[1] (**Figure** 1a) was amplified by PCR, to facilitate the addition of *Nco* I and *Nhe* I restriction sites to the 5′ and 3′ ends of the cDNA, respectively, and allow insertion into the pET30b Pharm'Xpress® *E. coli* vector. The vector was then used to transform *E. coli* BRL (DE3).

The fermentation process

For feasibility expression studies, bacteria were grown in auto induction medium for its ability to promote high density cell growth and automatic induction of protein expression from lac promoters (AIM medium—5% (w/v) yeast extract and 10% (w/v) tryptone) to a 1 L scale (24 h, 180 rpm, 37°C). Pharm'Xpress® medium supplemented with glucose/$MgSO_4$ solution (60% glucose, 3.6% yeast extract) was used in the scale up fermentation process at 37°C in the presence of 20% pO_2. A sample from an initial cell banking aliquot was used to produce an initial preculture of 25 mL. This was used to inoculate a second overnight pre-culture of 300 mL supplemented Pharm'Xpress® medium and incubated at 27°C with shaking at 180 rpm (initial optical density at 600 nm (OD_{600nm}) between 0.015 and 0.020). The temperature of this final preculture was increased to 37°C for the last 30 min to reach an OD_{600nm} within the range of 2.0-4.0. Three hundred millilitre was used to inoculate 2.7 L supplemented Pharm'Xpress® medium in a 3 L fermentor (Bioflow 3000, New Brunswick). A first fed batch (60% glucose, 3.6% $MgSO_4$) was initiated when all the glucose was consumed. When the culture reached OD 40.0, the feed was replaced by a second feed containing 30% glucose, 15% yeast extract. Isopropyl β-D-thiogalactopyranoside was added to 1 mM when the culture reached an OD_{600nm} of 60.0 and upon reaching an OD_{600nm} of 100.0 the culture was transferred to 15°C with 300 rpm agitation overnight (14 h).

Preparation of inclusion bodies

The fermentation broth was centrifuged at 5000 × *g* for 15 min and the pellet stored at −20°C until required. Two hundred and fifty milliliter of pellet was suspended in 50 mM Tris/HCl pH8.5 + 150 mM NaCl, 1 mM pepstatin, 1 mM leupeptin to obtain an OD_{600nm} of 140.0. Three cycles of cell lysis were performed at 1100 and 110 bar using a high pressure homogenizer (GEA Niro Soavi NS1001L, PANDA) followed by 1 h centrifugation at 15,000 × *g*. The supernatant was discarded.

The pellet was washed three times to prepare inclusion bodies for solubilisation. For the first wash 80 mL 50 mM Tris/HCl pH8.5 + 2M NaCl was used, for the second, 80 mL 50 mM Tris/HCl pH8.5 + 2% Triton X100 and finally 80 mL 50 mM Tris/HCl pH 8.5.

Solubilisation/purification

The washed pellet was solubilised overnight under constant stirring at 8°C in 40 mL 50 mM Tris/HCl pH 8.5 + 150 mM NaCl, 6 M Guanidine HCl and 20 mM dithiothreitol (DTT). The supernatant was then loaded on a gel filtration column (Superdex 200, GE Healthcare) and fractionated using an AKTA purifier system. Prior to loading, the column was equilibrated with one column volume of 50 mM Tris/HCl pH 8.5 + 150 mM NaCl, 6 M Guanidine HCl, 20 mM DTT. The fractions collected were analyzed on SDS-PAGE gel under denaturing and reducing conditions.

Refolding

The refolding process was adapted from a two-step refolding protocol for bovine chymotrypsinogen[16] and optimized for the maggot zymogen. Briefly, a 100-fold dialysis step was performed overnight with stirring at 4-8°C to remove the DTT using SpectraPor dialysis membrane (cut off 3.5 kDa) against 50 mM Tris/HCl pH8.5 + 150 mM NaCl, 6M Guanidine HCl. The protein concentration in the soluble fraction after the dialysis was determined (UV measurement at 280 nm) and adjusted to 1 mg/mL. A second 100-fold dialysis step was performed against 50 mM glycine/NaOH pH 9.5, 10 mM NaCl, 1.2 M Guanidine HCl. A third buffer exchange (100-fold) was performed with the soluble fraction of the previous step against 50 mM Tris/HCl + 10 mM NaCl, 1 mM $CaCl_2$, 150 mM sucrose, 0.0118 nM Tween 20 in an attempt to stabilize the protein.

Concentration and analysis of the ISP zymogen

The refolded proteins were concentrated ten fold using concentration units (Amicon, Millipore) cut off 3 kDa and the protein concentration determined by Bradford analysis and UV measurement. The molecular weight of the ISP zymogen was determined by electrospray ionisation time-of-flight mass spectrometry (see supplementary data IIa). Analytical gel filtration was carried out on S75 GE equilibrated with 50 mM Tris, pH 8.5, 10 mM NaCl, 0.0118 nM Tween 20, 1 mM $CaCl_2$, 150 mM sucrose and 10% glycerol

(see supplementary data IIb). Electrophoresis (SDS-PAGE) of ISP zymogen under denaturing and reducing conditions was conducted and the gel stained with Coomassie PAGE Blue protein staining solution (Thermo Scientific, R0571). The major bands were removed from the gel, protein extracted and N-terminal sequences determined.

Enzymatic activation of the recombinant zymogen

Activation of the zymogen is necessary to convert the chymotrypsinogen to the active chymotrypsin and was achieved using immobilised trypsin following the method in Ref. 1. Ten microliters of bovine trypsin-agarose (Sigma-Aldrich, T-1763) was washed five times with 1 mL phosphate buffered saline (PBS) pH7.2 before centrifuging the beads at 13,000 $\times$ *g* on a microfuge. All free liquid was removed with blotting paper and a freshly thawed 200 μL aliquot of 1.07 mg/mL zymogen added. The reaction mixture was incubated at 37°C for 1 h in an orbital incubator rotating at 240 rpm to ensure constant suspension of the beads. After centrifugation the supernatant containing the active chymotrypsin I (ISP) was decanted into a sterile 1.5 mL Eppendorf tube and transferred to ice prior to assay.

Assay of proteolytic activity

The conversion of the substrates succinyl-alanyl-alanyl-prolyl-phenylalanyl-aminomethyl coumarin (suc-ala-ala-pro-phe-AMC) and tosyl-glycyl-prolyl-arginyl-aminomethyl coumarin (tosyl-gly-pro-arg-AMC) indicated the presence of active enzyme.[1] Samples were assayed at 10 μg/mL in PBS, pH 7.2. Twenty microlitres of sample were added to 180 μL 11.111 μM substrate (final assay concentration of 10 μM) and incubated at 37°C. Substrate conversion (release of aminomethyl coumarin (AMC)) was measured on the fluorometer (excitation wavelength 355 nm, emission wavelength 460 nm) at time 0 and after 20 min. An AMC standard curve (0–10 μM) was included on the assay plate and enzyme activity expressed as quantity of product formed per μg ISP per minute (pmol/μg/min) at 37°C in 10 μM substrate. Proteolytic activity was further characterized by addition of the trypsin/chymotrypsin inhibitor phenylmethylsulphonyl fluoride (PMSF, 1 mM) and the trypsin inhibitor amidino-phenylmethylsulphonyl fluoride (APMSF, 50 μM). SDS-PAGE of ISP and ISP zymogen was conducted in the presence or absence of 0.1% human haemoglobin, under non-reducing or reducing conditions[17] and gels stained with Coomassie blue.

Stability testing

Stability of ISP. Activated ISP was incubated at 37°C for 72 h and sampled at 24 h intervals. Samples were tested for chymotryptic activity and run in a non-reduced state into a 0.1% haemoglobin gel.

Stability of Sf9-Derived Chymotrypsin. Activated Sf9-derived chymotrypsin[1] was filtered using a 0.22 μM nitrocellulose membrane and aliquotted into 50 μL lots in 150 μL Eppendorf tubes. Tubes were stored at room temperature (mean 20.3°C, min 18.0°C, max 23.5°C), 5°C (mean 5.0°C, min 4.0°C, max 6.0°C), −20°C (mean −24.0°C, min −24.4°C, max −20.0°C), −40°C (mean −41.4°C, min −43.0°C, max −39.0°C) and −80°C (mean −80.0°C, min −81.0°C, max −78.0°C) and sampled over a 1 year period. Samples were immediately tested for chymotryptic activity.

Degradation of wound eschar

Ethical and R&D approval for the collection of wound eschar was obtained from the University of Nottingham ethics committee (REC05/Q2424/194; QDE100502). Wound eschar was collected with the patients' written and informed consent from a non-healing venous leg ulcer before and during maggot therapy and stored at −20°C until required. Protein from wound eschar was homogenised into PBS and the insoluble material removed by centrifugation at 13000 $\times$ *g* for 10 min. Protein content was estimated using the Biorad protein assay and the solubilised protein stored at −20°C until required.

ISP (1 μg) was added to 25 μg wound eschar in the presence or absence of 50 μM APMSF at 37°C for 20 h, then electrophoresed under reducing conditions using SDS-PAGE.[1] ISP was also diluted in Normlgel® (Molnlycke) so that 100 μL of gel contained 1 μg of ISP. An equivalent volume of PBS was also mixed with Normlgel® as a control. One hundred microlitres of Normlgel® containing PBS or Normlgel® containing ISP was placed in the bottom of 1.5 mL Eppendorf tubes. 25 μg of PBS-extracted wound eschar protein was layered over the top of the Normlgel® preparations and incubated for 20 h at 37°C. Following incubation the eschar protein was removed, concentrated by acetone precipitation and degradation analysed by 12% SDS-PAGE followed by staining with Coomassie Blue.

Results

Analysis of the ISP zymogen

In a typical fermentation of the ISP, the growth rate obtained varied from $\mu = 2.2\ h^{-1}$ to $\mu = 0.3\ h^{-1}$. The process was continued until all the glucose in the medium was consumed (batch phase). During the first feed phase, glucose is added to maintain cell growth at a rate of between $\mu = 0.3\ h^{-1}$ to $\mu = 0.9\ h^{-1}$. A linear feed combining yeast extract and glucose started at OD_{600} 40 and induction of protein expression started at OD_{600} 60. During this phase, the growth rate varied from $\mu = 0.1\ h^{-1}$ to $\mu = 0.2\ h^{-1}$. The expression phase lasted for 6 h and the final OD_{600} obtained was ~110.

The equivalent of 1g of ISP was obtained from 1 L of culture fermentation process as determined just after the first step of purification (SEC-FPLC). Inclusion bodies were refolded in three dialysis steps. We observed that the largest proportion of the protein was lost during the reduction of the chaotropic agent concentration from 6 M guanidium-HCl to 1.2 M guanidium-HCl. These resulted in a recovery of approximately 66 mg/L of ISP at 1.1 mg/mL, which produced an overall yield (starting after the first purification step) of 6.6%.

Following activation with trypsin, below, a specific activity of 2458 pmol/min/μg ISP was recorded when tested against the chymotrypsin substrate. The ISP zymogen had a mass of 25451.8 Da on electrospray TOF mass spectrometry (see supplementary data IIa), and N-terminal sequencing (Edman) of the electrophoresed ISP zymogen resolving at approximately 25 kDa detected the MFEGRITNGQ sequence at the N-terminus of the expressed protein (**Figure** 1b).

Analytical gel filtration of ISP showed that the majority of the ISP zymogen resolved as a homogeneous, monomeric fraction with >90% purity (see supplementary data IIb). An elution volume (Ve) of 12.5 mL was recorded for ISP,

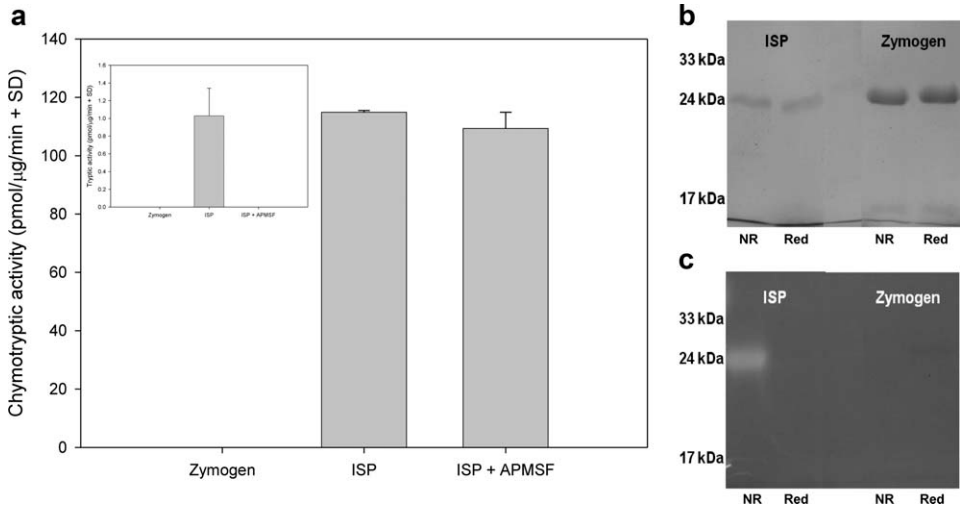

Figure 2. The chymotryptic and tryptic activities of activated recombinant maggot chymotrypsin (ISP).

Nonactivated zymogen and activated maggot chymotrypsin (ISP) were reacted with 11.1-μM suc-ala-ala-pro-phe-AMC [chymotryptic substrate (a)] or tosyl-gly-pro-arg-AMC (inset) for 20 min in the presence or absence of the trypsin inhibitor APMSF (50 μM). Nonactivated zymogen and activated maggot chymotrypsin (ISP) in the presence or absence of the trypsin inhibitor 50 μM APMSF. SDS-PAGE (b) SDS-PAGE 0.1% haemoglobin proteolytic activity gel (c) under nonreducing (NR) and reducing (Red) conditions.

between the ribonuclease (13.7 kDa/13.7 mL) and carbonic anhydrase (29 kDa/11.9 mL) standards. A minor peak, observed at Ve 8.6 mL, eluted at the exclusion limit of the column, and possibly represents aggregated ISP.

One minor contaminant was noted on SDS-PAGE, a protein with sequence homology to the bacterial chaperone lbpB. The level of contamination was substantially reduced in the 3L scale-up. There was some indication of low level endoproteolysis. The sequencing of a minor polypeptide was suggestive of proteolysis of ISP between residues 109/110.

Enzymatic activation of the recombinant zymogen

Activation and Chymotryptic Activity. Trypsin activation of the zymogen results in the removal of the peptide MFEGR from the zymogen. After activation by immobilised bovine trypsin, ISP displayed chymotryptic activity (**Figure** 2a; $P < 0.001$ Student's *t*-test), which was not significantly inhibited by the trypsin-specific inhibitor APMSF (50 μM). The non-activated zymogen displayed no chymotryptic activity. A trace (<1%) of tryptic activity (as defined by the conversion of the trypsin-specific substrate tosyl-gly-pro-arg-AMC) was present in the ISP after activation and this activity was inhibited by 50 μM APMSF (**Figure** 2a; inset $P < 0.001$ Student's *t*-test).

ISP Activity on SDS-PAGE. **Figure** 2b indicates that, upon activation, ISP zymogen showed a slight reduction in mass. The comparative banding seen when comparing the reduced and nonreduced ISP confirmed that the activated ISP was monomeric. Substrate gel analysis (**Figure** 2c) indicated that the zymogen lacked proteolytic activity, which appeared following tryptic activation. The reduced and alkylated ISP lacked activity.

Stability Testing of Activated ISP and Degradation of Wound Eschar. **Figure** 3 shows stability data for activated ISP. Chymotryptic activity (**Figure** 3a) was only slightly reduced over the period of incubation at 37°C. The difference was deemed to be insignificant based on a Kruskal-Wallis one-way ANOVA. The haemoglobin gel indicates consistent proteolytic activity over the same period (**Figure** 3b) and the SDS-PAGE gel indicates the persistence of the ISP (**Figure** 3c). Long-term stability testing of an activated research grade ISP expressed in Sf9 cells is shown for comparison in **Figure** 3d.

Figure 4a shows degradation of wound eschar after incubation with recombinant ISP when compared to activation buffer-treated wound eschar. The ISP retained activity in the presence of the trypsin-specific inhibitor APMSF (50 μM). When admixed into Normlgel® ISP retained its ability to digest wound eschar (**Figure** 4b).

Discussion

The production of a recombinant, active, cGMP grade *L. sericata* chymotrypsin I (ISP) is critical if this enzyme is to be considered suitable for development as a novel wound debridement/management product. Prior analyses of yeast, mammalian, and bacterial expression systems for chymotrypsinogen resulted in the selection of *E. coli* as the system of choice. The production of ISP was initiated using *E. coli* BLR (DE3) transformed with ISP-pET30b in a 3 L fermentor. Insoluble protein was extracted and inclusion bodies solubilised. Gel-filtration chromatography was used to initially purify the zymogen followed by enrichment by ion-exchange chromatography. The zymogen was refolded and concentrated 10-fold on a 3 kDa molecular weight cut off membrane. Analysis by mass spectrometry confirmed a mass of 25451.81 Da and this was further supported by SDS-PAGE. N-terminal sequencing of the ISP protein detected the MFEGRITNGQ sequence from the beginning of the polypeptide. The majority of the ISP zymogen resolved as a homogeneous (monomeric) fraction following analytical gel filtration chromatography.

The presence of heat shock protein lbpB was observed during batch mode culture but not during fed batch fermentation although we cannot completely rule out a low level of expression in the latter. This suggests that the different culturing conditions could have an impact on the expression levels of this protein. Fed batch fermentation could be considered as

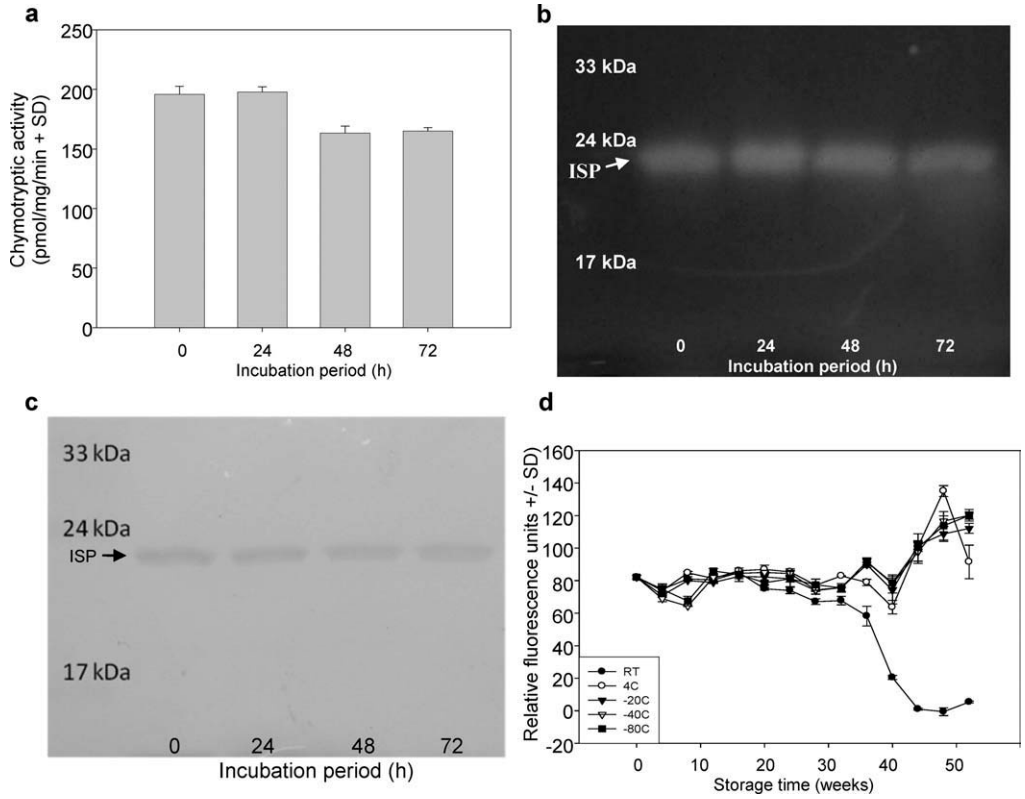

Figure 3. Stability testing of activated ISP.

Activated ISP incubated at 37°C and sampled at 24 h periods. Chymotryptic activities (a), and SDS-PAGE 0.1% haemoglobin proteolytic activity gel (b) and SDS-PAGE (c) under nonreducing conditions. Stability of activated Sf9 cell expressed chymotryptic activity during storage (d).

less stressful than batch mode thus the suggestion that the expression of lbpB is a stress response is well placed.

The expressed, refolded, and purified zymogen was activated by immobilised bovine trypsin. After activation, the recombinant protein demonstrated activity typical of a chymotrypsin, hydrolyzing only the chymotryptic substrate suc-ala-ala-pro-phe-AMC and not the trypsin substrate tosyl-gly-pro-arg-AMC. This activity was also inhibited in a dose dependent manner by PMSF, an inhibitor of trypsins and chymotrypsins, but not by APMSF, an inhibitor of trypsins only, further confirming the activity to be chymotryptic in nature and mirroring the activities of the recombinant material previously produced in insect cells.[1] The zymogen showed no chymotryptic activity. However, a trace (<1%) of tryptic activity (as defined by the conversion of the trypsin-specific substrate tosyl-gly-pro-arg-AMC) is present in the ISP after activation and this activity is inhibited by 50 μM APMSF, which suggests that this is bovine trypsin that has leached from the beads rather than nonspecific substrate conversion by the ISP. Leaching of ligands linked to matrices by cyanogen bromide linkage has been documented[18] and we have subsequently immobilized trypsin via an N-hydroxy-succinimide ester linkage which eliminates leaching. Historically, we have used a non-cGMP grade trypsin for the activation of the zymogen. The use of a commercially available cGMP grade trypsin will be required to realize a fully cGMP-compatible production process. A cGMP grade porcine trypsin is available and we can confirm that porcine trypsin does activate the chymotrypsin I zymogen (data not shown).

SDS-PAGE analysis of activated ISP and zymogen indicated proteins of molecular weights of around 25 kDa, whether reduced or non-reduced. This indicates that the ISP is a single polypeptide chain. We have also shown that the ISP is capable of digesting a haemoglobin substrate in situ

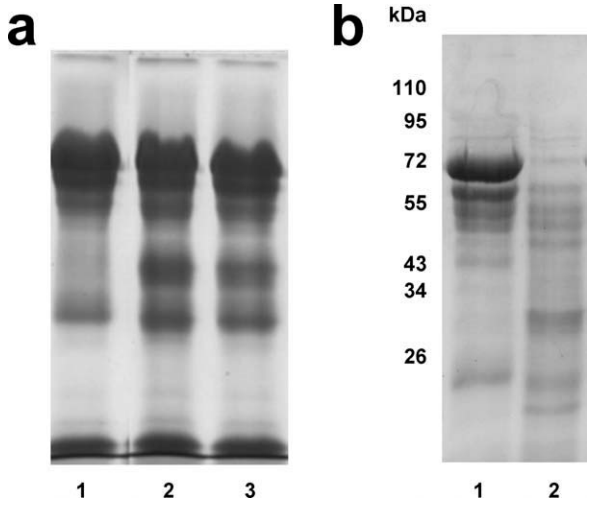

Figure 4. Enzymatic degradation of chronic wound eschar by ISP.

Activated ISP (1 μg) was incubated with 25 μg slough/eschar at 37°C for 20 h in the presence (lane 3) or absence (lane 2) of 50-μM APMSF and electrophoresed in a 12% SDS-PAGE gel under reducing conditions (a). Slough/eschar alone is shown in lane 1. Normlgel/ISP admix (1μg ISP in 100-μl Normlgel) and the equivalent Normlgel/PBS control were incubated with 25-μg slough/eschar from a venous leg ulcer at 37°C for 20 h (b). The supernatant was collected and electrophoresed in a 12% SDS-PAGE gel under reducing conditions. Lane 1 Normlgel/PBS, Lane 2 Normlgel/ISP.

in the gel. Its activity is associated with a molecular weight of ~25 kDa. Reduction of the ISP leads to a loss of activity, suggesting a requirement for internal disulphide bonding for activity. The recombinant activated enzyme was also shown to be stable in preliminary assay, and was effective in ex vivo slough/eschar debridement assays.

In conclusion, we have expressed *L. sericata* chymotrypsinogen I in *E. coli* and defined the conditions for the purification and refolding of the zymogen. Furthermore, the recombinant zymogen can be activated using immobilised bovine trypsin resulting in an active enzyme (ISP), capable of degrading patient wound eschar. As the defined product has been successfully expressed in a cGMP- acceptable bacterial system, we are in the position to be able to scale up to produce a cGMP grade protein for use in clinical trials. Furthermore, once scale up is achieved, we will be able to further formulate delivery systems, based on the prototypes already developed.[2,3]

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