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A Culture-Based Method for Determining the Production of Secreted Protease Inhibitors

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Abstract

We have developed a culture-based method for determining the production of secreted protease inhibitors. The assay utilizes standard proteolysis detection plates to support microbial growth followed by infiltrating the plate with a protease and subsequently detecting the remaining protein by trichloroacetic acid (TCA) precipitation, or by bromocresol green (BCG) or Ponceau S (PS) staining. The presence of a protease inhibitor can be observed in the form of a protected zone of protein around the protease inhibitor-producing strain. Using the protease inhibitors α -2-macroglobulin, aprotinin, leupeptin, and bestatin and the primary and secondary forms of *Photobacterium luminescens* in combination with the protease trypsin, we were able to demonstrate that the assay is specific for the cognate inhibitor of the protease and for bacteria secreting protease inhibitors. In addition, when casein-containing plates were used, the size of the diffusion zone was inversely correlated with the molecular weight of the inhibitor allowing a relative estimation of the protease inhibitor molecular weight. This assay is useful for detecting the presence of microbial secreted protease inhibitors and may reveal their production by microorganisms that were not previously recognized to produce them.

Keywords

Secreted protease inhibitors; *Photobacterium luminescens*; α -2-macroglobulin; aprotinin; leupeptin; bestatin

I. Introduction

Proteases secreted by bacteria, fungi and other microbes are widely distributed in nature and play roles in nutrient acquisition and pathogenesis (Winn *et al.* 2006). Simple, readily available methods exist for determining the presence of secreted proteases such as gelatin

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and casein hydrolysis plates that are visualized with trichloroacetic acid (TCA) precipitation or dyes such as bromochreosol green (BCG) (Medina and Baresi, 2007; Vijayaraghavan and Vincent, 2013). Due to the simplicity of these and other protease tests, the presence of proteases are known for various microorganisms in sufficient detail to be able to ascribe the percentages of a given species that are positive for extracellular proteolysis. For example, only 2% of *Pantoea agglomerans* are protease positive, whereas 50% of *Photorhabdus luminescens* and more than 90% of *Proteus vulgaris* are protease positive (Table 6–7 of Winn et al., 2006).

The presence of and role for bacterial protease inhibitors is not well understood (Kantyka et al., 2010). Most bacterial protease inhibitors are intracellular or periplasmic, such as the *Escherichia coli* protein ecotin (Maurizi, 1992). Ecotin is a highly stable serine protease inhibitor of trypsin, chymotrypsin, chymase and pancreatic elastase that is also capable of inhibiting factor Xa (Seymour et al., 1994), kallikrein, factor XIIa, (Ulmer et al., 1995) and neutrophil elastase (Eggers et al., 2004). Because ecotin homologues are present in pathogenic organisms such as *Rickettsia*, *Pseudomonas* and *Burkholderia* and inhibit neutrophil elastase in addition to pancreatic elastase (Eggers et al., 2004), it is thought that ecotin family members may play a protective role against the innate cellular immune system (Kantyka et al., 2010). However, the nonpathogenic gut bacterium *Bifidobacterium longum* also produces a protease inhibitor of elastase-like serine proteases (Ivanof et al., 2006). Other protease inhibitors such as the staphostatins of *Staphylococcus aureus* and *S. epidermidis* are thought to have the function of protecting the cytoplasm from proteolysis during the production of secreted cysteine proteases produced by these species (Kantyka et al., 2011). Overall, it seems likely that bacterial protease inhibitors play a variety of roles among different bacterial species.

Only a subset of the known bacterial protease inhibitors are secreted into the media, for example those of *Bacillus brevis* (Shiga et al., 1992; 1995), *Prevotella intermedia* (Grenier 1994) and *Photorhabdus (Xenorhabdus) luminescens* secondary (phase variant) form (Wee et al., 2000). The roles of these protease inhibitors is also unknown, but are likely to modulate external proteolytic degradation one or more ways that benefits the microorganisms that produce them. In some cases the protease inhibitors may be regulating proteases secreted by the organism itself. For example, *Photorhabdus luminescens* primary form secretes a protease (Schmidt et al., 1988), while the secondary form secretes both a protease and a protease inhibitor of its own protease (Wee et al., 2000). It is also possible that some secreted protease inhibitors are directed toward proteases of other organisms. Recent advances in surveying the human microbiota and its collective microbiome suggest there are many complex microbial interactions that can either regulate beneficial bacterial population structures or contribute toward microbial dysbiosis (Grice and Segre, 2011; Eloe-Fadrosh and Rasko, 2013). Protease inhibitors could potentially be a factor in inter-bacterial species interactions through the inhibition of proteases, or even regulate host proteolytic functions (Sánchez et al., 2010). Interestingly, a new approach to treatment of inflammatory bowel disease is the introduction of a probiotic *Lactococcus casei* expressing a secreted form of the protease inhibitor elafin (Motta et al., 2012). Heterologous expression of secreted protease inhibitors by bacterial vectors such as tumor-targeted *Salmonella*

delivering anticancer proteins (Pawelek et al., 1997; Low et al., 1999; Bermudes et al., 2002) could also result in increased efficacy from these therapeutic agents due to reduced proteolysis of the effector proteins within the proteolytic environment of the tumor. There has been no readily available culture-based assay for the presence of secreted protease inhibitors and thus knowledge of the presence of naturally occurring strains is limited. The availability of a petri plate-based method for identification of bacterial strains producing secreted protease inhibitors and its application to the analysis of microbiome and environmental communities should facilitate better understanding of the potential roles of these inhibitors in commensal, mutualistic and dysbiotic associations as well as within environmental communities.

Protease inhibition assays generally couple a biochemical test for proteolysis with purified fractions that potentially contain protease inhibitors. The protease inhibition assays conducted by Wee et al. (2000) for identification of the *P. luminescens* protease inhibitor utilized ammonium sulfate precipitation of the culture supernatant, redissolution of the precipitate in phosphate buffer, dialysis, separation by isoelectric focusing, and then testing of fractions for inhibitory activity using azocoll (Chavira et al., 1984). Assay of the *Prevotella intermedia* protease inhibitor also utilized a similar approach beginning with ammonium sulfate precipitation of the culture supernatant, redissolution and dialysis, and then followed by size exclusion gel filtration. Inhibitory activity was monitored by determining hydrolysis of the chromogenic peptide N- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). Determination of *Bacillus brevis* protease inhibitor activity (Shiga et al., 1992) was also determined from redissolved ammonium sulfate precipitates, with protease activity and its inhibition monitored by following the change in absorbance at 275 nm of the protease substrate N ^{α} -*p*-tosyl-L-argininemethylester.

We explored modification of protease detection plates described by Medina and Baresi (2007) and Vijayaraghavan and Vincent (2013) for their use in detecting the reverse function of proteolysis, that of protease inhibition. We accomplished this by using the same or similar media formulations described by these authors, growing the bacteria on them, exposing the test plates to a solution containing a protease, and then detecting zones of protein protection using protein binding dyes or TCA protein precipitation.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used were *Photobacterium* (*Xenorhabdus*) *luminescens* strain Hm primary and secondary forms (K. H. Nealson, University of Wisconsin, Milwaukee), *Citrobacter freundii* (ATCC #33128), *Escherichia coli* B (CGSC 5713) and the following clinical isolates (provided by N. Bishop, California State University, Northridge, Northridge, CA), *Staphylococcus aureus*, *S. epidermidis* and *Enterococcus faecalis*. The strains were maintained on tryptic soy agar and were allowed to grow two days at 30°C under ambient air conditions using the protease/protease inhibitor media described below. Liquid suspensions (2 μ l) were applied to the plates for the protease inhibitor studies described below

2.2. Chemical and Microbiological Reagents

Unless otherwise noted, standard chemical reagents were from Sigma-Aldrich (St. Louis, MO) and microbiological media components were from Becton Dickinson (Sparks, MD).

2.3. Dye-Containing Casein Media

The base casein media was that of Vijayaraghavan and Vincent (2013) that contained 5 grams of peptone, 1.5 grams of yeast extract, 1.5 grams of sodium chloride per liter, except that 0.5% casein was used (instead of 1.0%) and dissolved as described by Montville (1983) using 0.02 M NaOH. When bromochresol green (BCG) was added directly to the media, 0.0015% was used. We used both BCG preincorporated into the media as well as post incubation addition of a BCG dye reagent for 1–2 hrs containing 0.028% BCG dissolved in 0.56% (w/v) succinic acid, 0.1% (w/v) NaOH (Vijayaraghavan and Vincent; 2013) with 0.6% Brij-35 (Durgawale et al, 2005) acidified to pH 4.2 with HCl. This dye reagent was utilized following exposure to a protease inhibitor or bacterial growth and subsequent exposure to a protease. After primary staining and then marking the agar with a 23 ga syringe needle dipped in India ink at a corresponding register marked on the plastic petri plate, the agar was destained in water by completely removing it from the lower plastic dish. We also utilized Ponceau S (PS) staining by flooding the plate with 5 ml of the stain containing 0.1% PS and 5% acetic acid in water for 1–2 hrs, removing the agar from the plate and destaining overnight in water. The plates were then observed for the presence and absence of stained protein.

2.4. Dye-Containing Gelatin Media

The base gelatin media was that of Medina and Baresi (2007) which used 40 grams per liter of tryptic soy agar powder without glucose modified to contain 8 g of gelatin (instead of 16g) per liter. Approximately 5 ml of 20% trichloroacetic acid (TCA; Thermo Fisher, Waltham, MA) was used for protein precipitation per petri plate. We also combined this media with preincorporated BCG, or post-incubation BCG dye reagent or PS, each as described above for the casein plates.

2.5. Protease Inhibition Detection Protocol

The basic protocol was the combination of 3 steps (Figure 1) described further below. Because the protocol is destructive to the plate assayed, a duplicate or replica plate should be generated and retained for further analysis of the strains if necessary.

2.5.1. Exposure of the protease detection plate to a protease inhibitor or bacterial growth (Step 1)—We used either commercially available protease inhibitor peptides or growth of bacterial strains on the protease detection plates. Protease inhibitors were α -2-macroglobulin (7.8 mg/ml in sterile water; Thermo Fisher), aprotinin (0.3 mM in sterile dH₂O; Thermo Fisher), leupeptin (10 mM in sterile dH₂O; Thermo Fisher), and bestatin (1 mM in methanol; Thermo Fisher) that were pipetted as 2.0 μ l drops onto the surface of the protein containing plates and allowed to be absorbed into the plate and diffuse for 1 hour. The bacterial strains *Photorabdus (Xenorhabdus) luminescens* strain Hm primary and secondary forms, *Escherichia coli* B, *Citrobacter freundii*, *Staphylococcus aureus*, *S.*

epidermidis and *Enterococcus faecalis* were allowed to grow two days at 30°C. Following incubation, the bacterial strains were washed off the plate using a gentle stream of water in order to remove the colonies and eliminate their potential for surface inhibition effects on diffusion of the protease and/or dyes.

2.5.2. Exposure to a protease-containing solution (Step 2)—Preliminary studies showed that casein-containing plates required a higher concentration of trypsin to achieve clearing: we investigated trypsin concentrations as high as 2.5mg/ml. For most of the experiments, the plates were flooded with either 1.0 ml of sterile-filtered 0.625 mg/ml trypsin or 0.0625 mg/ml trypsin in 10 mM Tris 1mM EDTA pH 8.0 for casein and gelatin respectively. Following trypsin adsorption (20–60 min.), an additional 1 ml of sterile filtered water containing 2.5 mg carbenicillin and 750 µg streptomycin was also allowed to absorb into the plate in order to stop any further bacterial growth on plates that had contained bacterial colonies. The trypsin or trypsin/antibiotics were then allowed to incubate at 37°C overnight.

2.5.3. Detecting zones of protease inhibition (Step 3)—The zones of protease inhibition were observed by the presence of opaque or dye-stained zones surrounded by clearing as detected by either TCA precipitation, BCG previously incorporated into the media, or by the addition BCG or PS dyes. Radial diffusion was measured in triplicate, entered into Microsoft Excel, plotted, and analyzed using linear regression.

Results

Utilizing the protease inhibitors α -2-macroglobulin, aprotinin and leupeptin we established the general protocol shown in Figure 1. A comparison of the different variations on protein substrates, dyes and destains is shown in Table 1. Following the addition of a protease inhibitor and its diffusion into the plate, the majority of the protein contained within the plates was subsequently hydrolyzed by the addition of the protease trypsin. However, in the presence of the protease inhibitors, a zone of protection against proteolysis was created. When the plates were observed for the presence of protein either by trichloroacetic acid (TCA) precipitation or the localized concentration of a dye such as bromochrosol green (BCG) or Ponceau S (PS), a zone of unhydrolyzed protein was observed in the location of the protease inhibitor. Using α -2-macroglobulin, aprotinin, leupeptin and bestatin we found that we were only able to faintly visualize the α -2-macroglobulin and aprotinin on the casein plates with preincorporated BCG (Figure 2A). However, the combination of BCG-containing plates further stained with PS were the most sensitive when 0.625 mg/ml trypsin was used for casein and 0.0625 mg/ml trypsin was used for gelatin (Table 1; Figure 2B). Using those plates and staining procedure we were able to visualize all three of the trypsin inhibitors α -2-macroglobulin, aprotinin, and leupeptin, with no staining for bestatin. We also observed differences in a white ring of protein precipitation that occurred strongly on the gelatin plates when higher concentrations of trypsin (2.5mg/ml) were used, but only slightly on the casein plates when they were treated with the same trypsin concentration (Figure 3).

We also noticed on the casein plates, but to a lesser degree on the gelatin plates, that the zone of inhibition for leupeptin (molecular weight = 427 Da) was notably larger than the zone of inhibition for aprotinin (molecular weight = 6511 Da), or that of the α -2-macroglobulin (molecular weight ~720 kDa) which had the smallest diffusion zone (Figure 2). These results thereby suggested the assay might provide a means of determining the relative molecular weight of unknown inhibitors. When the radius was measured and plotted against the inverse of the molecular weights, a general relationship of size and diameter was also apparent for the casein plates and to a lesser degree for the gelatin plates (Figure 4). A linear R^2 regression analysis was performed using Microsoft Excel, which resulted in an $R^2 = 0.73$ for the casein plates and an $R^2 = 0.47$ for the gelatin plates (Figure 4).

Analysis of the six bacterial strains, *Photorabdus luminescens* Hm primary and secondary forms, *Citrobacter freundii*, *Staphylococcus aureus*, *S. epidermidis* and *Enterococcus faecalis* using the casein plates with preincorporated BCG followed by PS staining showed that the *Photorabdus luminescens* Hm secondary form, and not the primary form, was protease inhibitor positive (Figure 5). The *P. luminescens* secondary form colony grew fairly large (Figure 5A), and it was apparent that the inhibitor only diffused a short distance from the edge of the colony. Likewise, the α -2-macroglobulin also diffused only a short distance. We sometimes observed that the protease inhibitor secreted by *P. luminescens* secondary form appeared to diffuse wider than the α -2-macroglobulin, although this was not observed consistently (data not shown). We also found that unlike the casein plates (Figure 5), when gelatin plates were used (Table 1, variations 8, 9, 11 and 12), *E. coli* resulted in giving a positive protease inhibitor signal (data not shown).

Discussion

There is a paucity of knowledge about the presence and distribution of microbial secreted protease inhibitors, despite widespread knowledge of microbial secreted proteases. This may be due to the lack of a readily available protease inhibitor assay, although it is also possible that secreted protease inhibitors are in fact uncommon. The method described here is both easy and useful for determining the presence of secreted protease inhibitors. In our protease inhibitor control studies that we used to develop the assay, we utilized both trypsin inhibitors (α -2-macroglobulin, aprotinin and leupeptin) and a non-trypsin metalloprotease inhibitor (bestatin). Because no zone of inhibition was observed for bestatin, the results were specific for the cognate inhibitor of the protease added, which in our assays was the enzyme trypsin. The *Photorhabdus luminescens* secondary form secreted protease inhibitor is also known to be an inhibitor of trypsin (Wee et al., 2000) and was the only organism that gave a visibly positive result on casein plates. These results were therefore consistent with previous results of Bleakley and Neelson (1988) and Schmidt et al., (1988) who first described the *P. luminescens* protease and Woo et al, (2000) who first described the protease inhibitor on the secondary form, which validates our approach for detecting secreted protease inhibitors. Based on the N-terminal peptide sequence from Woo et al. (2000) it is likely that the *P. luminescens* protease inhibitor corresponds to the *Photorhabdus temperata* hypothetical protein PTE_01760. That peptide is 137 amino acids in length and corresponds to a molecular weight of 15.7 kDa, but the monomeric or multimeric nature of that protein has not yet been described and therefore its apparent molecular weight is unknown.

By comparing relative diffusion zones of protease inhibitors, different inhibitors could potentially be recognized, and although our best linear regression R^2 value (0.73) was not significant, an overall correlation with relative size differences was observed. However, the method can only be considered to provide an approximation of relative molecular weight, and had the best R^2 value when casein plates were used. The low molecular weights of the leupeptin and aprotinin inhibitors used in the study and their larger diffusion zones make them less useful for determining the relative molecular weight of higher molecular weight proteins produced by bacteria over the two day growth period than the larger molecular weight α -2-macroglobulin. Use of additional higher molecular weight protease inhibitor standards may produce more useful estimations for the higher molecular weight protease inhibitors.

The assay can detect protease inhibitors of single or multiple bacteria of a mixed population. The ability to assay multiple bacteria on a single plate is a significant advantage and provides a means to discover new bacterial strains that produce secreted protease inhibitors from a variety of natural samples. Expression of secreted protease inhibitors by bacteria and other microbes and our ability to detect them may have several important consequences. We suspect that application of this method to individual strains, environmental samples or microbiota samples is likely to greatly broaden our knowledge of their presence and distribution among taxa and may yield clues to understanding the various roles secreted protease inhibitors may play, including contributions to stable commensalistic and mutualistically symbiotic microbiological associations. It is possible that either protease inhibitor excess or insufficiency contributes to dysbiosis. Microbial delivery of a human therapeutic protease inhibitor (Motta et al., 2012) represents an attempt to restore gut proteolysis associated with inflammatory bowel disease to a balanced status. We also postulate that tumor-targeted *Salmonella* vectors delivering heterologous anticancer proteins within the proteolytic environment of the tumor would have increased efficacy due to reduced proteolysis of the therapeutic proteins mediated by expression of genetically protease inhibitors. Protease inhibitors expressed by tumor-targeted bacteria may also have direct antitumor and/or antimetastasis effects, as many tumor proteases are known to contribute to tumor metastasis (Edwards et al., 2008).

There were a number of minor variations we tried involving the protein substrates, the preincorporation of and post-addition of protein detecting dyes and TCA precipitation (Table 1). Not all of these methods were equally capable of detecting secreted protease inhibitors and indicates that adapting this method to different substrates and conditions to investigate microbes with varying physiologies may require additional adjustments. While most of the methods were able to detect protease inhibitors, we found the highest contrast and greatest sensitivity with secondary staining using PS which worked best with casein. We also found that when gelatin plates were used, *E. coli* resulted in giving a positive signal (data not shown). It is possible that conditions we did not control for such as slight acidity caused the release of ecotin, which might correlate with its potentially protective role when the bacteria are internalized by phagocytes. We also recognize that false positive results could occur if a bacterium secretes a high concentration of protein that is not degraded by the added protease, and thus would appear as a zone of protein surrounding the bacterium. In

this regard, we noted that when high concentrations of the α -2-macroglobulin protease inhibitor was used, we were able to stain the inhibitor itself using the PS in plates where no other protein had been added to the agar, although not nearly the same magnitude of staining that was observed with the inhibitor-created zone of protection using protein-containing plates. Overall the assay represents a relative shift in protein concentration rather than indicating an absolute absence or presence since the protease itself (e.g., trypsin) is also present and protease resistant, and likely contributes to a low background of protein staining.

Another potential limitation of this method is interference by proteases secreted by a mixed population bacteria being assayed on the same petri plate. If such a protease diffuses into the region of another bacterium producing a protease inhibitor, no zone of protease inhibition may be detected if that particular protease is not also inhibited by the protease inhibitor, thereby resulting in a false negative result.

Among the other observations we noted is that the presence of a protease inhibitor combined with a protease can give counterintuitive effects. In the case of the gelatin plate when a higher concentration of trypsin was used, the presence of aprotinin resulted in a ring of white precipitation just outside of the PS strained protein (Figure 3). This is likely due to the initial phenomena of protein precipitation caused by the protease giving the plate a cloudy appearance in that area due to separation of a hydrophilic portion of the protein from the hydrophobic one, resulting in precipitation of the hydrophobic portion. The appearance of this effect was transient, which may have been due to the continued action of the protease completely hydrolyzing the protein that was initially precipitated (data not shown). Similarly, the presence of a protease inhibitor can give the appearance of clearing at the site of the protease inhibitor if the protein incorporated into the plate is translucent, such as low concentrations of gelatin (data not shown). This is also likely to be due to the initial phenomena of protein precipitation by the protease, leaving a relatively clear protected inner circle.

To investigate the full spectrum of naturally occurring secreted protease inhibitors, all of the classes of proteases must be analyzed and would require appropriate protein substrates that are sensitive to those proteases. Our study demonstrated the specificity of the inhibitor for the protease added to the protease detection plates. While we did not investigate other proteases in developing this method, the availability of cognate protease inhibitors for serine, cysteine, aspartic and metalloprotease proteases provide the necessary tools and controls to expand the assay to include other classes of proteases and protease inhibitors.

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Highlights

- We use several different protease detection systems modified to determine the production of secreted protease inhibitors.
- We demonstrate the specificity of the cognate protease inhibitors for the protease.
- We allow a relative estimation of the molecular weight of the protease inhibitor.
- The method can be used to identify bacteria secreting protease inhibitors.
- The method can be used to study mixed bacterial populations.

Figure 1.
Stepwise procedure for detecting protease inhibitors (PIs).

Figure 2.

Protease inhibitor zones of protein protection. Protease inhibitors were pipetted onto the surface of a casein plate containing bromochreosol green (BCG). After 1 hour the plate was flooded with 1.0 ml of sterile-filtered 0.625 mg/ml trypsin and allowed to absorb, followed by addition of 1 ml carbenicillin/streptomycin and incubation at 37°C overnight for plates that had contained bacterial colonies. **A)** BCG containing plate prior to Ponceau S (PS) staining and **B)** The same plate following PS staining and destaining. 1) α -2-macroglobulin (2 μ l of 7.8 mg/ml in dH₂O), 2) aprotinin (2 μ l of 0.3 mM in sterile dH₂O), 3) leupeptin (2 μ l of 10 mM in sterile dH₂O), and 4) bestatin (2 μ l of 1 mM in methanol).

Figure 3.

Protease inhibitor precipitation zone. The aprotinin protein protection zones were visibly distinct under conditions of increased amounts of trypsin on the different protein containing plates. (A) Aprotinin (2 μ l of 0.3 mM in sterile dH₂O) with 2.5mg/ml trypsin on a bromochreosol green (BCG) gelatin plate and (B) aprotinin (2 μ l of 0.3 mM in sterile dH₂O) with 2.5mg/ml trypsin on a BCG casein plate.

Figure 4.

Correlation of the protease inhibitor molecular weight with the diffusion zone that creates the zone of protein protection. The inhibition zones created by the three trypsin inhibitors were plotted as the radius against the inverse molecular weights of the inhibitors (α -2-macroglobulin, (~720 kDa; aprotinin, 6511 Da; and leupeptin, 427 Da). R^2 linear regression was inserted using Microsoft Excel. Dotted line, bromochreosol green (BCG) gelatin; solid line, BCG casein.

Figure 5.

Bacterial protease inhibitor zones of protein protection. The bacteria were grown for 2 days at 30°C on casein plates with bromochreosol green (BCG). The bacteria were gently washed off and the plate flooded with 1.0 ml of sterile-filtered 0.625 mg/ml trypsin and allowed to absorb, followed by addition of 1 ml carbenicillin/streptomycin and incubation at 37°C overnight. The plate was further stained with Ponceau S (PS) and then destained in water overnight. **A)** Growth pattern of the colonies on the BCG plate containing casein following incubation at 30°C. **B)** PS staining pattern of the same plate. 1) *Photobacterium luminescens* 1° form, 2) *P. luminescens* 2° form, 3) *Citrobacter freundii* B, 4) *Staphylococcus epidermidis*, 5) *S. aureus*, 6) *Enterococcus faecalis*, α2m) α-2-macroglobulin.

Table 1

Protease inhibitor detection efficiency, protein substrates, dyes and destaining.

Variation	Protein Substrate	Dye incorporated in plate	Dye or Reagent added after growth	Destain	Relative Detection Efficiency		
					a-2 Macroglobulin	Aprotonin	Leupeptin
1	0.5% Casein	None	BCG	1 hour Water	+	+/-	+/-
2	0.5% Casein	None	Ponseau S	Overnight Water	+++	+++	+++
3	0.5% Casein	None	TCA	15 min development	+	+/-	+/-
4	0.5% Casein	BCG	BCG	1 hour Water	+	+	+/-
5	0.5% Casein	BCG	Ponseau S	Overnight Water	+++	+++	+++
6	0.5% Casein	BCG	TCA	15 min development	+/-	+/-	+/-
7	0.8% Gelatin	None	BCG	1 hour Water	-	-	-
8	0.8% Gelatin	None	Ponseau S	Overnight Water	+++	+++	+++
9	0.8% Gelatin	None	TCA	15 min development	+++	+++	+++
10	0.8% Gelatin	BCG	BCG	1 hour Water	-	-	-
11	0.8% Gelatin	BCG	Ponseau S	Overnight Water	+++	+++	+++
12	0.8% Gelatin	BCG	TCA	15 min development	+++	+++	+++