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(54) Title: PROLYL ENDOPEPTIDASE PROBES

(57) Abstract: Prolyl endopeptidase (PE) activity in lung samples is detected by contacting the lung sample with a probe comprising a -P-X- (or -X-P-, -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and (b) detecting the signal.



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Prolyl Endopeptidase Probes

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Assignee: SRI International

[001] This work was supported by grants No. R21AI085402 from the NIAID; the Government has certain rights in this invention.

[002] This application claims priority to US Ser No. 61/382,315, filed Sep 13, 2010.

[003] **Field of the Invention:** The field of the invention is a prolyl endopeptidase probes and methods of use.

[004] Background of the Invention

[005] Poor diagnosis is a major problem when deciding treatment options for patients suffering from infectious diseases. Sensitive and specific assays for the reliable detection of existing and emerging pathogens are needed.

[006] The invention involves using a peptide based probe which when added to a biological sample of an infected patient, results in signal generation (e.g. fluorescence). This invention enables identification and preparation of specific peptide-based fluorogenic probes that can be used in a fluorescence based assay to detect signature proteolytic activity in the biological fluids of human hosts. We have established proteolytic activity as a biomarker for rapid and accurate assessment of individuals' health status.

[007] The invention provide an entirely new class of in vitro diagnostics to detect a variety of infectious diseases with high sensitivity and specificity. Health care providers can effectively use this invention as a part of their diagnostics platform. The probes can also be used to monitor therapy, wherein early diagnosis and treatment could cut mortality in half for many infectious diseases.

[008] Aspects of this disclosure were published by the inventors in: Watson et al., BioTechniques 51, 95-104 (Aug 2011) and Watson et al., PLoS ONE Jun 2011, 6, 6, e21001.

[009] Summary of the Invention

[010] The invention provides methods of detecting prolyl endopeptidase (PE) activity in a lung sample.

[011] In one embodiment the method comprises the steps of: (a) contacting the sample with a probe comprising a -P-X- (or -X-P- or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and (b) detecting the signal.

[012] In particular embodiments:

[013] -the probe comprises an -P-X-P-X- or -P-X-P-X-P- recognition site, particularly wherein X is a residue that is not a prolyl bioisostere;

[014] -X is a chiral (non-G) amino acid;

[015] -X is N, F, Y, S, H or G;

[016] -X is a natural or unnatural L- or D- amino acid, preferably G, A, V, L, I, M, F, W, P, S, T, C, Y, N, Q, D, E, K, R, or H;

[017] -X is a prolyl bioisostere flanked on each side by N, F, Y, S, H or G;

[018] -X is a prolyl bioisostere flanked on each side by a natural or unnatural L- or D- amino acid, preferably G, A, V, L, I, M, F, W, P, S, T, C, Y, N, Q, D, E, K, R, or H;

[019] -the prolyl bioisostere is selected from proline, dehydroproline, homoproline, N-methylamino acid, and decahydroisoquinoline carboxylate, each of which may be optionally substituted;

[020] - X is aminoluciferin;

[021] -the probe further comprises a hydrophilic moiety and the probe is water soluble;

[022] -the probe is internally quenched;

[023] -one end of the recognition site is operably-linked to a first chromophore (such as a FRET donor or a fluorophore), and the other end of the recognition site is operably-linked to a second, different chromophore (such as a FRET acceptor or a quenching moiety), particularly wherein the chromophores are independently operably-linked to the recognition site through a linker that is glycine, serine, a peptide of serine and/or glycine, a mini-PEG (8-amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid;

[024] -proteolysis of the probe produces a substrate, and the sample is further contacted with an enzyme that reacts with the substrate to produce the signal;

[025] -the sample is bronchoalveolar lavage fluid of a patient having a lung disease, particularly a lung infection that is a fungal infection, a bacterial infection, a parasitic infection, or a viral infection;

[026] -the lung has a disease that is a non-infectious inflammatory disease, including chronic or acute inflammatory disease; and/or

[027] -the contacting and detecting steps are repeated with different PE probes, particularly with different lung samples, wherein the method determines one or more differences in PE activity between the samples.

[028] The invention also provides methods of detecting prolyl endopeptidase (PE) activity in each of a plurality of samples. In one embodiment the method comprises the steps of: (a) contacting each sample with a panel of different probes each comprising a -P-X- (or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and (b) detecting and comparing the resultant signals from each sample to determine differences in PE activity between the samples.

[029] The invention also provides probes adapted to the subject methods. In one embodiment the invention provides an internally quenched fluorogenic probe (IQFP) for prolyl endopeptidase (PE) activity comprising a -P-X (or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, wherein one end of the recognition site is operably-linked to a FRET donor, and the other end of the recognition site is operably-linked to a FRET acceptor, wherein PE hydrolysis of an amide bond of the recognition generates an optical signal.

[030] In particular embodiments: the donor and acceptor are independently operably-linked through a linker that is glycine, serine, a peptide of serine and/or glycine, a mini-PEG (8-amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid.

[031] The invention also provides methods of making and using the subject probes in the disclosed methods, including diagnostic, characterization and screening methods.

[032] The invention provides all combinations and subcombinations of recited particular embodiments as if each combination and subcombination had been specifically, separately recited.

[033] Brief Description of the Figures

[034] Fig. 1. General structure of fluorogenic probes for detection of invasive aspergillosis.

[035] Fig. 2. BALF IQFP assay: PNP

[036] Fig. 3. BALF IQFP assay: PYP

[037] Fig. 4. BALF assay: PYP

[038] Fig. 5. BALF v. Serum

[039] Fig. 6. Infected v. Uninfected

[040] Fig. 7. (A) Amino acid distribution of sequences cleaved by BALF; (B) Amino acid distribution of sequences cleaved by serum; (C) Motifs cleaved by BALF; (D) Motifs cleaved by serum; (E) Distribution of BALF protease cleavage sites; (F) Distribution of BALF serum cleavage sites.

[041] Detailed Description of Specific Embodiments of the Invention

[042] In one embodiment the invention provides a peptide based probe comprising a protease substrate that has a specific sequence and consists of 3 to 10 amino acids. On either side of this peptide sequence is located a chromophore and/or a fluorophore. This probe remains optically silent when added to a biological fluid of a healthy patient, but when added to a biological fluid of an infected patient it emits a fluorescence signal, typically with emission the range of 300 nm to 800 nm. Assayable biological fluids include serum and bronchoalveolar lavage fluid, as well as urine, blood, plasma, and saliva. Lung-derived samples may be obtained from biopsy samples, BALF, etc. The probes can be used for the detection of fungal diseases, such as invasive aspergillosis, as well as bacterial diseases (e.g. tuberculosis, MRSA), parasitic diseases (e.g. Leishmaniasis, Chagas disease), and viral diseases (e.g. H1N1, SARS).

[043] The invention is generally applicable in the in vitro diagnostics industry. Specifically, a panel of multiple fluorogenic probes can be used to detect a variety of pathogenic conditions in parallel. The probes can also be used for detecting the level of infection (e.g. invasion vs. localization), response to antimicrobials (e.g. recovery), and to diagnose the general health status of individuals.

[044] In a particular embodiment the invention provides detection of protease activity by luminescence, for the diagnosis of infection or other uses, e.g. using a peptide based probe which when added to a biological sample of an infected patient, followed by sequential addition of a recombinant enzyme (such as luciferase) results in generation of luminescence.

The peptide based probe consists of a protease substrate that has a specific sequence and consists of 3 to 10 amino acids. The probe contains a luminescent substrate, such as aminoluciferin, either within the substrate sequence or in flanking regions at the N- or C-terminus. Cleavage of the substrate by one or more proteases results in liberation of the luminescent substrate, which is chemically modified by a recombinant enzyme, resulting in the generation of light. This probe remains optically silent when added to a biological fluid of a healthy patient, but when added to a biological fluid of an infected patient it emits a luminescent signal with emission anywhere in the range of 300 nm to 800 nm. The probes are useful for the detection of fungal diseases, such as invasive aspergillosis, as well as bacterial diseases (e.g. tuberculosis, MRSA), parasitic diseases (e.g. Leishmaniasis, Chagas disease), and viral diseases (e.g. H1N1, SARS).

[045] A specific example of this aspect is the probe sequence GGGPAlucPGGKK, where Aluc corresponds to aminoluciferin and all other letters correspond to natural amino acids according to the single letter code. Cleavage of this sequence by an endopeptidase generates the peptide fragment AlucPGGKK. Subsequent cleavage by a protease liberates the Aluc moiety, which is converted to aminooxyluciferin by the addition of luciferase enzyme, resulting in the generation of light.

[046] In a particular embodiment the invention provides imaging pulmonary infection or inflammation with protease substrate probes, e.g. using imaging techniques to identify specific areas of infection or inflammation within the lungs in vivo to facilitate targeted therapy or surgery. The invention provides an imaging agent comprising a protease substrate sequence attached to a reporter moiety. The substrate sequence corresponds to a protease that is present during a specific infection, such as invasive aspergillosis, or an inflammatory condition, such as cystic fibrosis. The imaging agent is administered by the pulmonary or intravenous route. Cleavage of the substrate sequence in the presence of a specific protease results in activation of the attached reporter. Examples of reporter modalities include fluorescence, luminescence, PET, MRI, and SPECT, among others. This aspect is useful for determination of specific localized sites of infection or inflammation within the lung for many diseases, including pneumonia, bronchiectasis, emphysema, tuberculosis, fibrosis, chronic obstructive pulmonary disease, and asthma.

[047] In a particular embodiment the invention provides peptides of the sequence X-P-X-P-X as biomarkers for infection or inflammation. We have found that proteases that generate peptides of the sequence proline-glycine-proline are elevated in pulmonary inflammatory conditions, such as invasive aspergillosis and cystic fibrosis. The presence of short peptide

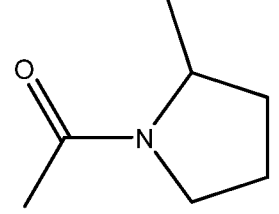
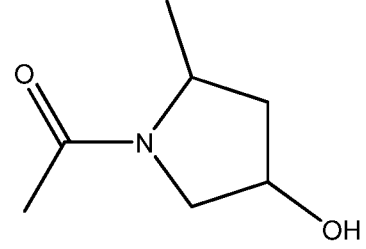
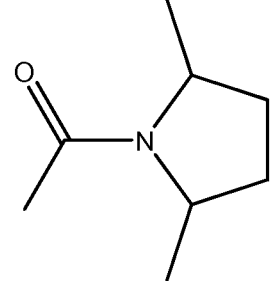
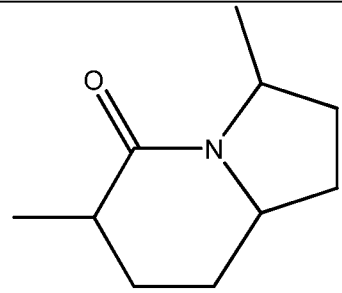
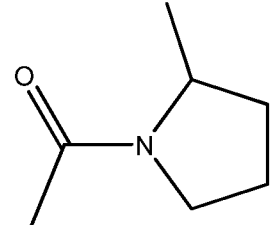
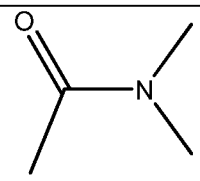
fragments produced by these proteases provide useful biomarkers for the presence or extent of disease. Useful peptides include sequences that include X-P-X-P-X, where P corresponds to proline and X corresponds to any sequence of natural or unnatural amino acids.

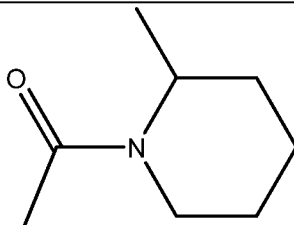
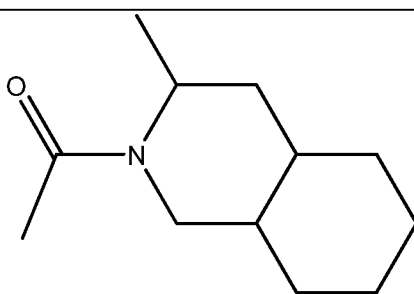
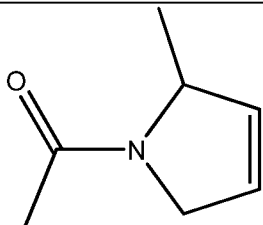
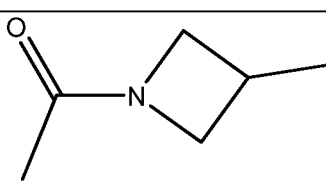
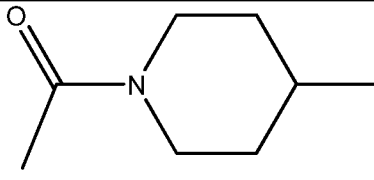
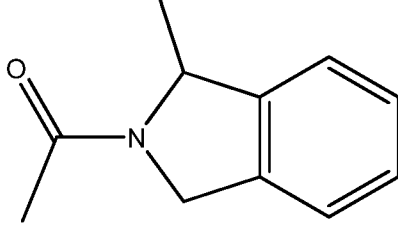
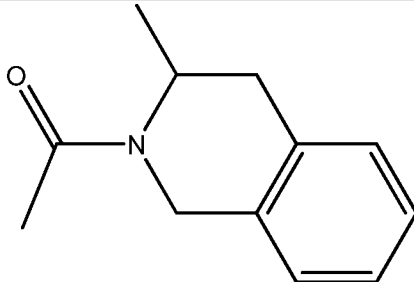
[048] In one aspect the invention provides a method of detecting prolyl endopeptidase (PE) activity in a lung sample, comprising the steps of: (a) contacting the sample with a probe comprising a -P-X- (or -X-P- or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and (b) detecting the signal.

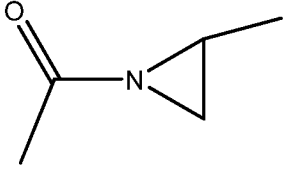
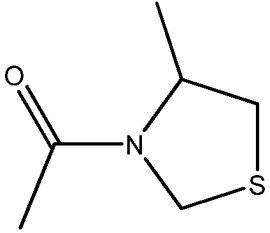
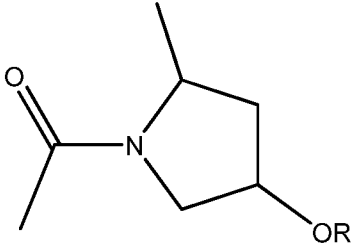
[049] In particular embodiments the probe comprises a -P-X-, -X-P-, -P-X-P-, -P-X-P-X- or -P-X-P-X-P- recognition site, wherein X is a residue that is not a prolyl bioisostere, and is preferably a non-proline natural amino acid, particularly, N, F, Y, S, H or G. X can also be a residue incorporatable in a peptide through flanking amide bonds. Such amino acid alternatives may be used to modulate its stability, solubility or other functionalities. In a particular embodiment the residue contributes to the signal, such as providing a signal generating enzyme substrate, e.g. is aminoluciferin.

[050] Prolyl bioisosteres are well-established structural and functional analogs of proline (e.g. Sampognaro et al., *Bioorg Med Chem Lett.* 2010 Sep 1;20(17):5027-30), and those used herein are substitutable for proline in peptide probes for prolyl endopeptidase. The prolyl bioisosteres may be generated from alternative scaffolds, such as proline, cyclopentene, cyclohexene, pyrrolidine, pyrrolidinone, carbocyclic, acyclic groups and 4-phenyl-2-carboxypiperazine (e.g. Nilsson et al., *J Comb Chem.* 2001 Nov-Dec;3(6):546-53; Thorstensson 2005, *Linköping Studies in Science and Technology, Dissertations No. 990*). In particular embodiments the prolyl bioisostere is selected from proline, homoproline, hydroxyproline, dehydropoline, aminoproline, 5,6-benzohomoproline (Shuman et al. *J Org Chem* 1990, 55, 738-41), alkylproline, N-methylamino acid, and decahydroisoquinoline carboxylate, each of which may be optionally substituted with hydroxy, halogen, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, substituted or unsubstituted arylalkyl, substituted or unsubstituted amine, substituted or unsubstituted alkylamine, substituted or unsubstituted dialkylamine, substituted or unsubstituted alkoxy, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted aryloxy, substituted or unsubstituted alkoxy, substituted or unsubstituted haloalkyl, benzo, NO₂, CF₃, aryl, carboxyl, cyano, isocyanate, or alkoxycarbonyl.

[051] Examples of suitable prolyl bioisosteres include:

	1-(2-methylpyrrolidin-1-yl)ethanone
	1-(4-hydroxy-2-methylpyrrolidin-1-yl)ethanone
	1-(2-methylpyrrolidin-1-yl)ethanone
	3,6-dimethylhexahydroindolizin-5(1 <i>H</i>)-one
	1-(optionally-substituted, 2-methylpyrrolidin-1-yl)ethanone
	<i>N,N</i> -dimethylacetamide

	1-(2-methylpiperidin-1-yl)ethanone
	1-(3-methyloctahydroisoquinolin-2(1H)-yl)ethanone
	1-(2-methyl-2,5-dihydro-1H-pyrrol-1-yl)ethanone
	1-(3-methylazetidin-1-yl)ethanone
	1-(4-methylpiperidin-1-yl)ethanone
	1-(1-methylisoindolin-2-yl)ethanone
	1-(3-methyl-3,4-dihydroisoquinolin-2(1H)-yl)ethanone

	1-(2-methylaziridin-1-yl)ethanone
	1-(4-methylthiazolidin-3-yl)ethanone
	1-(2-methyl, 4-R-oxy-1-yl)ethanone

[052] In particular embodiments the probe further comprises one or more additional flanking sequences to modulate its stability, solubility, or other functionality. In particular embodiments the probe comprises a hydrophilic moiety, such as polylysine (e.g. KKK) to increase water solubility.

[053] In particular embodiments the probe is internally quenched, particularly wherein one end of the recognition site is operably-linked to a fluorescence resonance energy transfer (FRET) donor (or a fluorophore), and the other end of the recognition site is operably-linked to a FRET acceptor (or a quenching moiety). In other embodiments, one end of the recognition site is operably-linked to a first chromophore, and the other end of the recognition site is operably-linked to a second chromophore, wherein fluorescence emission of one chromophore is quenched by the other chromophore, which may be by FRET, aggregation, or other mechanism of contact or non-contact quenching. In particular embodiments, the donor and acceptor (or fluorophore and quenching moiety, or first and second chromophores) are independently operably-linked through a linking residue that is glycine, serine, a peptide of serine and/or glycine, a mini-PEG (8-Amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid (e.g. 6-aminohexanoic acid). A variety of alternative FRET structures may be incorporated to modulate signaling or other functionality of the probe; for example, in particular embodiments the probe comprises a plurality of donors and/or a plurality of acceptors.

[054] In particular embodiments the sample is bronchoalveolar lavage fluid of a patient having a lung disease, such as a lung infection or lung inflammation, particularly lung

inflammation as a results of an acute or chronic health conditions, autoimmune disease or trauma, and particularly in immuo-compromised patients. In particular embodiments the lung and/or patient has a fungal infection (e.g. invasive aspergillosis), a bacterial infection (e.g. tuberculosis, MRSA), a parasitic infection (e.g. leishmaniasis, chagas disease), or a viral infection (e.g. H1N1, SARS). In other embodiments, the lung and/or patient has a disease that is a non-infectious inflammatory disease, including chronic (e.g. chronic obstructive pulmonary disorder, chronic asthma, bronchiectasis, chronic bronchitis, emphysema, pulmonary fibrosis) or acute (trauma, acute asthma, hypersensitivity) inflammatory disease.

[055] The probe signal is detected by convenient means, depending on the selected probe structure including optical signal detection (e.g. colorimetric, luminescent or fluorescence-based assay) mass detection (e.g. mass spectroscopy) or nuclear detection (e.g. NMR, radiography). A variety of assay formats may be employed, including ELISA, RIA, chromatography, microscopy, etc.

[056] The invention also provides methods of using libraries or panels of probes to characterize and/or diagnose disease, particularly wherein the contacting and detecting steps are repeated with different PE probes, particularly with different lung samples, wherein the method determines one or more differences in PE activity between the samples. Hence, the invention provides methods of identifying probes that specifically detect PE activity as biomarkers of a disease condition, particularly in a lung sample, and methods of identifying probes that distinguish between two or more distinct prolyl endopeptidase enzymes.

[057] In particular embodiments the invention provides methods of detecting prolyl endopeptidase (PE) activity in each of a plurality of samples. In one embodiment the method comprises the steps of: (a) contacting each sample with a panel of different probes each comprising a -P-X- (or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and (b) detecting and comparing the resultant signals from each sample to determine differences in PE activity between the samples.

[058] The invention also provides probes adapted to the subject methods. In one embodiment the invention provides an internally quenched fluorogenic probe (IQFP) for prolyl endopeptidase (PE) activity comprising a -P-X (or -P-X-P-) PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an

amide bond, wherein one end of the recognition site is operably-linked to a FRET donor, and the other end of the recognition site is operably-linked to a FRET acceptor, wherein PE hydrolysis of an amide bond of the recognition generates an optical signal.

[059] In particular embodiments: the donor and acceptor are independently operably-linked through a linking residue that is glycine, polyglycine or a mini-PEG (8-amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid (e.g. 6-aminohexanoic acid).

[060] The invention also provides methods of making and using the subject probes in the disclosed methods, including diagnostic, characterization and screening methods.

[061] Examples

[062] Example 1. Specific examples of unique fluorogenic probes for detection of invasive aspergillosis

[063] Specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "MeOCGly-G-G-X1-X2-X3-G-G-Dap(Dnp)-K-K" where MeOCGly corresponds to 7-methylcoumarin-4-acetyl glycine, Dap(Dnp) corresponds to diaminopropionyl(dinitrophenyl), and X1-X3 correspond to variable natural amino acids as depicted in Fig. 1. All other letters correspond to natural amino acids according to standard single letter code:

- 1) MeOCGly-G-G-S/T-I/L-F/Y-G-G-Dap(Dnp)-K-K
- 2) MeOCGly-G-G-S/T-I/L-N/Q-G-G-Dap(Dnp)-K-K
- 3) MeOCGly-G-G-I/L-F/Y-F/Y-G-G-Dap(Dnp)-K-K
- 4) MeOCGly-G-G-A/V-I/L-I/L-G-G-Dap(Dnp)-K-K
- 5) MeOCGly-G-G-P-A/V-N/Q-G-G-Dap(Dnp)-K-K
- 6) MeOCGly-G-G-P-A/V-P-G-G-Dap(Dnp)-K-K
- 7) MeOCGly-G-G-P-K/R-P-G-G-Dap(Dnp)-K-K
- 8) MeOCGly-G-G-P-D/E-K/R-G-G-Dap(Dnp)-K-K
- 9) MeOCGly-G-G-P-D/E-P-G-G-Dap(Dnp)-K-K
- 10) MeOCGly-G-G-P-N/Q-A/V-G-G-Dap(Dnp)-K-K
- 11) MeOCGly-G-G-P-N/Q-P-G-G-Dap(Dnp)-K-K
- 12) MeOCGly-G-G-P-I/L-P-G-G-Dap(Dnp)-K-K
- 13) MeOCGly-G-G-P-S/T-A/V-G-G-Dap(Dnp)-K-K
- 14) MeOCGly-G-G-P-S/T-P-G-G-Dap(Dnp)-K-K
- 15) MeOCGly-G-G-P-S/T-F/Y-G-G-Dap(Dnp)-K-K

16) MeOCGly-G-G-P-F/Y-A/V-G-G-Dap(Dnp)-K-K

17) MeOCGly-G-G-P-F/Y-K/R-G-G-Dap(Dnp)-K-K

18) MeOCGly-G-G-P-F/Y-N/Q-G-G-Dap(Dnp)-K-K

19) MeOCGly-G-G-P-F/Y-P-G-G-Dap(Dnp)-K-K

[064] Additional specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "MeOCGly-X-Dap(Dnp)-K-K" where MeOCGly corresponds to 7-methylcoumarin-4-acetyl glycine, Dap(Dnp) corresponds to diaminopropionyl(dinitrophenyl), and X corresponds to a sequence of natural amino acids as specified according to the standard single letter code:

- 1) MeOCGly- GGPGPGGDap(DNP)KK -NH₂
- 2) MeOCGly- GGPFPGG Dap(DNP)KK -NH₂
- 3) MeOCGly- GGPYPGG Dap(DNP)KK -NH₂
- 4) MeOCGly- GGPNPGG Dap(DNP)KK -NH₂
- 5) MeOCGly- GGPQPGG Dap(DNP)KK -NH₂
- 6) MeOCGly- GGPHPGG Dap(DNP)KK -NH₂
- 7) MeOCGly- GGSPPGG Dap(DNP)KK -NH₂
- 8) MeOCGly- GGPDPGG Dap(DNP)KK -NH₂
- 9) MeOCGly- GGPPPGG Dap(DNP)KK -NH₂
- 10) MeOCGly- GGPPFPGG Dap(DNP)KK -NH₂
- 11) MeOCGly- GGPNPNPGG Dap(DNP)KK -NH₂
- 12) MeOCGly- GGPNPGPNPGG Dap(DNP)KK -NH₂
- 13) MeOCGly- GGPYPYPGG Dap(DNP)KK -NH₂
- 14) MeOCGly- GGPYPGPYPGG Dap(DNP)KK -NH₂
- 15) MeOCGly- GGNFAGG Dap(DNP)KK -NH₂
- 16) MeOCGly- GGNFVGG Dap(DNP)KK -NH₂
- 17) MeOCGly- GGNYAGG Dap(DNP)KK -NH₂
- 18) MeOCGly- GGNYVGG Dap(DNP)KK -NH₂
- 19) MeOCGly- GGQFAGG Dap(DNP)KK -NH₂
- 20) MeOCGly- GGQFVGG Dap(DNP)KK -NH₂
- 21) MeOCGly- GGQYAGG Dap(DNP)KK -NH₂
- 22) MeOCGly- GGQYVGG Dap(DNP)KK -NH₂

[065] Additional specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "5(6)FAM-X-Lys(5(6)TMR-K-K" where 5(6)FAM corresponds to 5(6)-carboxyfluorescein, Lys(5(6)TMRcorresponds to

Lysine(5(6)-tetramethylrhodamine), and X corresponds to a sequence of natural amino acids as specified according to the standard single letter code:

- 1) 5(6)FAM- GGGPGPGG Lys(5(6)TMR)KK -NH₂
- 2) 5(6)FAM- GGGPFPGG Lys(5(6)TMR)KK -NH₂
- 3) 5(6)FAM- GGGPQPGG Lys(5(6)TMR)KK -NH₂
- 4) 5(6)FAM- GGGPNPGG Lys(5(6)TMR)KK -NH₂
- 5) 5(6)FAM- GGGPYPGG Lys(5(6)TMR)KK -NH₂
- 6) 5(6)FAM- GGGPNPNPGG Lys(5(6)TMR)KK -NH₂
- 7) 5(6)FAM- GGGPNPGPNPGG Lys(5(6)TMR)KK -NH₂

[066] Additional specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "1,5EDANS-X-Lys(DABCYL)-K-K" where 1,5EDANS corresponds to 5-(2-Aminoethylamino)-1-naphthalenesulfonic acid, Lys(DABCYL) corresponds to Lys(4-(dimethylaminoazo)benzene-4-carboxylic acid), and X corresponds to a sequence of natural amino acids as specified according to the standard single letter code:

- 1) 1,5EDANS- GGPNNPGG Lys(DABCYL) KK -NH₂
- 2) 1,5EDANS- GGPYPGG Lys(DABCYL) KK -NH₂
- 3) 1,5EDANS- GGPNNPNPGG Lys(DABCYL) KK -NH₂
- 4) 1,5EDANS- GGPNNPGPNPGG Lys(DABCYL) KK -NH₂

[067] Additional specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "Abz-X-Dap(Dnp)-K-K" where Abz corresponds to o-aminobenzoic acid, Dap(Dnp) corresponds to diaminopropionyl(dinitrophenyl), and X corresponds to a sequence of natural amino acids as specified according to the standard single letter code:

- 1) Abz- GGGPNPGG Dap(Dnp) KK -NH₂
- 2) Abz- GGGPYPGG Dap(Dnp) KK -NH₂
- 3) Abz- GGGPNPNPGG Dap(Dnp) KK -NH₂
- 4) Abz- GGGPNPGPNPGG Dap(Dnp) KK -NH₂

[068] Additional specific examples of unique fluorogenic probes for detection of invasive aspergillosis include the following sequences in the format of "DANSYL-X-Lys(5(6)FAM)-K-K" where DANSYL corresponds to 5-dimethylamino-1-naphthalenesulfonic acid, Lys(5(6)FAM) corresponds to Lysine(5(6)-carboxylfluorescein, and X corresponds to a sequence of natural amino acids as specified according to the standard single letter code:

- 1) DANSYL- GGGPNPGG Lys(5(6)FAM)KK -NH₂

- 2) DANSYL- GGGPYPPGG Lys(5(6)FAM)KK -NH₂
- 3) DANSYL- GGGPNPNPPGG Lys(5(6)FAM)KK -NH₂
- 4) DANSYL- GGGPNPGPNPPGG Lys(5(6)FAM)KK -NH₂

[069] Example 2. Secreted Proteases of *Aspergillus fumigatus* (AF) as Targets for Diagnosis of Invasive Aspergillosis (IA)

[070] Background: Innovative approaches are needed for rapid and accurate diagnosis of IA. We are investigating AF secreted proteases as novel diagnostic targets. The AF genome encodes up to 100 secreted proteases, many of which are expressed in vivo during infection. We hypothesize that internally quenched fluorogenic probes (IQFPs) derived from fungal protease substrates can be used to detect the enzymatic activity of AF proteases in the serum and bronchoalveolar lavage fluid (BALF) of infected patients, utilizing the unique thermotolerance of AF enzymes to distinguish them from host proteases.

[071] Methods: The substrate specificity of AF in vitro culture supernatant was profiled with a combinatorial IQFP library in comparison with human serum to identify fungus-specific substrates. An established guinea pig model of IA was used to collect BALF during active disease for in vivo protease substrate profiling.

[072] Results: In vitro protease substrate profiling identified approximately 75 IQFPs that were cleaved strongly by AF culture supernatant (greater than 4x fluorescence enhancement) but were not cleaved by human serum. A subset of IQFPs selected for further analysis corresponded to serine proteases that retained proteolytic activity up to 50 °C. Protease substrate profiling of BALF from AF-infected guinea pigs revealed several probes that are cleaved preferentially during infection, which are promising diagnostic candidates.

[073] Conclusions: Although IQFPs have long been used to image protease activity in vivo in oncology and inflammation, this may be the first demonstration of protease profiling for in vitro diagnosis of infection. The technology represents a promising alternative for diagnosis of IA and other blood-borne and pulmonary pathogens.

[074] Example 3. Early diagnosis of Invasive Aspergillosis (IA)

[075] Methods: Guinea pig inhalation model comprises neutropenia by cortisone acetate and cyclophosphamide (days -2 and +3), 1.2×10^8 conidia/mL nebulized AF in inhalation chamber, and serum and BALF obtained at predetermined time points; Vallor et al.

Antimicrob. Ag. Chemother. 2008, 52.

[076] Results: Proline-containing targets: PXP probes significantly diagnostic (fold ration >2.0) of infected BALF, followed by P-F/Y-X, P-S/T-X and P-X-A/V. Figs. 1-5.

[077] Conclusions: Cleavage of P-X-P substrates by infected guinea pig BALF at Day 7 post-infection is repeatable and highly significant; sensitivity and specificity at Day 7 are comparable to or better than existing assays (81%, 90%); and cleavage occurs primarily at P-XP and PX-P (human and fungal prolyl endopeptidases cleave at P-XP).

[078] Example 4. Identification of protease substrates as differential diagnostic biomarkers in lung fluid and serum

[079] Methods: Guinea pig inhalation model comprises neutropenia by cortisone acetate and cyclophosphamide (days -2 and +3), 1.2×10^8 conidia/mL nebulized AF in inhalation chamber, and serum and BALF obtained at predetermined time points; Vallor et al.

Antimicrob. Ag. Chemother. 2008, 52.

[080] Results: The protease substrate specificities of serum and BALF from guinea pigs were determined. Differences in the proteolytic fingerprints of the two fluids were striking: serum proteases cleaved substrates containing cationic residues and proline, whereas BALF proteases cleaved substrates containing aliphatic and aromatic residues. Notably, cleavage of proline-containing substrates was virtually absent in BALF derived from healthy, uninfected guinea pigs. Figs. 5-7.

[081] Conclusions: Substrate specificity profiling can complement existing proteomics techniques in assessment of differences in protease specificity between complex samples. Measurements of abundance alone may not be sufficient for identification of diagnostic or therapeutic targets, because the identified proteases may be inactive or proteolytically degraded. Once proteolytic specificities of interest are identified, the responsible proteases can be identified through the use of active site- directed probes designed from the known substrate specificity, This Example represents a simple but robust method for mapping the proteolytic specificities of complex biological fluids. Substrates identified using this method may serve as sensors for diagnosis and imaging purposes, scaffolds for focused synthesis of substrates and inhibitors, probes in the design of robust assays for inhibitors, and triggers for controlled drug delivery.

Table 1. Wells enhanced in infected guinea pigs

Plate	Well	Fold enhancement	Fold infected	Fold uninfected	FI infected	FI uninfected	Sequence (#)	Sequence (code)	Sequence
4	B6	2.13	2.18	1.02	325	188	324	P-393-352	P-A/V-N/Q
4	D6	2.62	2.85	1.09	488	234	326	P-393-P	P-A/V-P
4	D7	2.37	2.88	1.22	498	213	334	P-351-P	P-K/R-P
4	H7	2.02	1.64	0.81	161	118	338	P-350-351	P-D/E-K/R
4	D8	2.31	2.3	1	349	213	342	P-350-P	P-D/E-P
4	G8	3.33	4.02	1.21	394	151	345	P-352-393	P-N/Q-A/V
4	D9	2.76	3.93	1.43	598	261	350	P-352-P	P-N/Q-P
4	D10	2.21	2.74	1.24	474	223	358	P-407-P	P-I/L-P
4	G11	2.39	4.28	1.8	467	176	369	P-223-393	P-S/T-A/V
4	D12	2.81	3.63	1.29	665	277	374	P-223-P	P-S/T-P
4	F12	3.08	3.31	1.07	397	146	376	P-223-354	P-S/T-F/Y
5	A1	2.04	3.06	1.5	493	294	377	P-354-393	P-F/Y-A/V
5	B1	2.06	2.09	1.01	238	151	378	P-354-351	P-F/Y-K/R
5	D1	2.15	2.8	1.31	286	154	380	P-354-352	P-F/Y-N/Q
5	F1	4.07	4.71	1.16	424	124	382	P-354-P	P-F/Y-P

Table 2. Summary of results for screening probes against *Aspergillus fumigatus* infected and uninfected guinea pig bronchoalveolar lavage fluid.

Peptide Probes	<i>Aspergillus fumigatus</i> Strain	Infected BALF			Uninfected BALF			%FE	P values	Sensitivity	Specificity
		N	Mean Fold change	SD	N	Mean Fold Change	SD				
PNP*	AF293	16	5.68	3.11	16	1.52	0.70	274.00	0.0001	0.69	0.97
	CEA10	22	13.68	10.13	19	3.88	2.34	252.39	0.0001	0.95	0.42
PYP*	AF293	16	5.42	3.00	16	1.79	1.00	203.00	0.0001	0.69	0.97
	CEA10	21	14.62	13.89	17	3.56	1.95	310.44	0.0016	0.76	0.53
PFP	AF293	22	6.58	9.44	23	2.01	2.45	227.09	0.0377	0.60	0.91
	CEA10	25	12.44	11.35	20	2.92	1.86	326.24	0.0003	0.80	0.75
PHP	AF293	22	4.47	4.48	23	1.40	0.79	219.69	0.0043	0.41	0.91
	CEA10	25	10.61	9.51	20	3.34	2.27	217.59	0.0009	0.80	0.55
PSP	AF293	20	12.49	29.69	22	3.05	1.30	309.39	0.1716	0.75	0.64
	CEA10	23	11.00	8.05	20	4.54	3.05	142.55	0.0012	0.91	0.30
PNPNP	AF293	20	13.47	26.98	19	2.96	1.41	355.02	0.0979	0.75	0.68
	CEA10	23	8.72	5.54	20	4.13	2.39	111.20	0.0010	0.83	0.40

[082] Table 2 Legend. Variable region (Xaa-Yaa-Zaa) of the top 6 peptide probes are all listed in single amino acid code; AF293 and CEA10 are the two strains of *Aspergillus fumigatus* infected guinea pig BALF samples screened in this study.

[083] N stands for number of guinea pig BALF samples screened against the given peptide probe. Guinea pig infected and uninfected samples with very low background (t= 0 hour) and end point values (t= 6 hour) (i.e., no greater than 100 units difference between t=0 hour and t=6 hour) were not selected for the statistical calculations.

[084] Mean fold change values is the average of fluorescence fold change calculated for each guinea pig sample screened against respective peptide probe. Fluorescence fold change values are calculated according to the formula:

Fold Change = Fluorescence measured at time t= 6 hour / Fluorescence measured at time t=0 hour.

* Fold change values for these peptide probes were calculated at time t= 3 hour as the end point value.

[085] SD corresponds to standard deviation calculated for the given probe screened against the respective BALF sample.

[086] Percentage fluorescence efficiency (% FE) is calculated for each probe as follows,

$$\% \text{ FE} = 100 * [(\text{Fold change}_{\text{Infected BALF}} - \text{Fold change}_{\text{Uninfected BALF}}) / \text{Fold change}_{\text{Uninfected BALF}}]$$

Fold change values are all the mean fold change values.

[087] P values are calculated with two tailed student t-test with two sample unequal variance for each peptide probe screened against respective infected and uninfected BALF samples.

[088] Sensitivity and Specificity of the assay for each peptide probe was calculated as shown below,

Sensitivity = True positives/ (True positives+ False negatives)

Specificity = True negatives / (True negatives + False positives)

True positive = Fold change 3 or greater in infected guinea pig sample

True negative = Fold change less than 3 in uninfected guinea pig sample

False positive = Fold change 3 or greater in uninfected guinea pig sample

False negative = Fold change less than 3 in infected guinea pig sample.

[089] The descriptions of particular embodiments and examples are offered by way of illustration and not by way of limitation. All publications and patent applications cited in this specification and all references cited therein are herein incorporated by reference as if each individual publication or patent application or reference were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of detecting prolyl endopeptidase (PE) activity in a lung sample, comprising:
 - (a) contacting the sample with a probe comprising a -P-X- or -P-X-P- PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and
 - (b) detecting the signal.
2. The method of claim 1 wherein the probe comprises an -P-X-P-X- PE recognition site.
3. The probe of claim 1 wherein X is N, F, Y, S, H or G.
4. The probe of claim 1 wherein X is a prolyl bioisostere flanked on each side by N, F, Y, S, H or G.
5. The probe of claim 1 wherein the prolyl bioisostere is selected from optionally-substituted proline, homoproline, hydroxyproline, dehydropyrolidine, aminoproline, 5,6-benzohomoproline, alkylproline, N-methylamino acid, and decahydroisoquinoline carboxylate.
6. The method of claim 1 wherein X is aminoluciferin.
7. The method of claim 1 wherein the probe further comprises a hydrophilic moiety and the probe is water soluble.
8. The method of claim 1 wherein the probe is internally quenched.
9. The method of claim 1 wherein one end of the recognition site is operably-linked to a first chromophore, and the other end of the recognition site is operably-linked to a second chromophore that quenches the first chromophore.
10. The method of claim 1 wherein one end of the recognition site is operably-linked to a FRET donor, and the other end of the recognition site is operably-linked to a FRET acceptor, and the donor and acceptor are independently operably-linked through a linker that is glycine,

serine, a peptide of serine and/or glycine, a mini-PEG (8-amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid.

11. The method of claim 1 wherein proteolysis of the probe produces a substrate, and the sample is further contacted with an enzyme that reacts with the substrate to produce the signal.

12. The method of claim 1 wherein the sample is bronchoalveolar lavage fluid of a patient having a lung disease.

13. The method of claim 1 wherein the lung has a disease that is a lung infection that is a fungal infection), a bacterial infection, a parasitic infection, or a viral infection.

14. The method of claim 1 wherein the lung has a disease that is a non-infectious inflammatory disease.

15. The method of claim 1 wherein the lung has a disease that is invasive aspergillosis.

16. The method of claim 1 wherein the contacting and detecting steps are repeated with different PE probes.

17. The method of claim 1 wherein the contacting and detecting steps are repeated with different PE probes with different lung samples, wherein the method determines one or more differences in PE activity between the samples.

18. A method of detecting prolyl endopeptidase (PE) activity in each of a plurality of biological or physiological samples, comprising:

(a) contacting each sample with a panel of different probes each comprising a -P-X- or -P-X-P- PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, under conditions wherein PE activity of the sample specifically hydrolyzes an amide bond of the recognition site to generate an optical signal; and

(b) detecting and comparing the resultant signals from each sample to determine differences in PE activity between the samples.

19. An internally quenched fluorogenic probe (IQFP) for prolyl endopeptidase (PE) activity comprising a -P-X- or -P-X-P- PE recognition site, wherein P is a prolyl bioisostere, X is a residue that is not a prolyl bioisostere or is a prolyl bioisostere flanked on each side by a residue that is not a prolyl bioisostere, and "-" is an amide bond, wherein one end of the recognition site is operably-linked to a first chromophore, and the other end of the recognition site is operably-linked to a second chromophore, wherein PE hydrolysis of an amide bond of the recognition generates an optical signal.

20. The probe of claim 19 wherein the chromophores are independently operably-linked to the recognition site through a linker that is glycine, serine, a peptide of serine and/or glycine, a mini-PEG (8-Amino-3,6-dioxaoctanoic acid or 11-amino-3,6,9-trioxaundecanoic acid) or a linear aliphatic alpha-amino acid (e.g. 6-aminohexanoic acid).

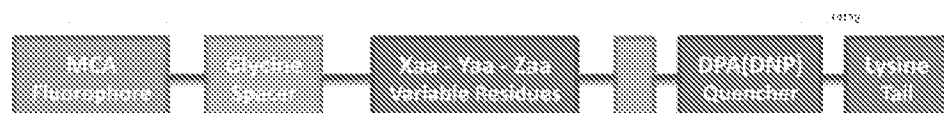


Fig. 1

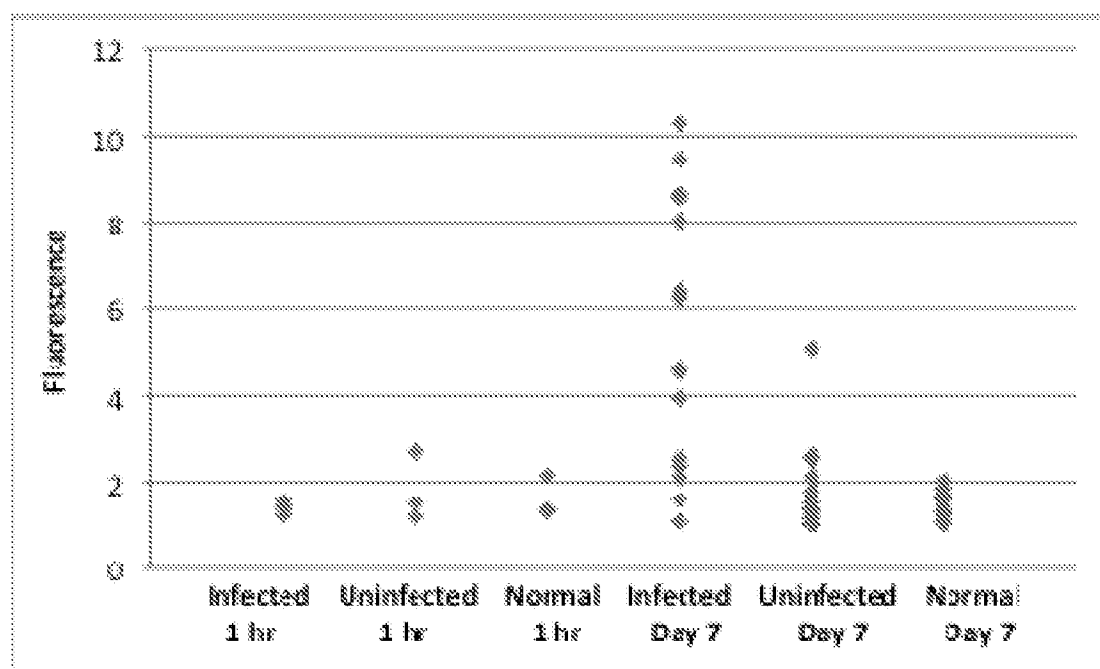


Fig. 2

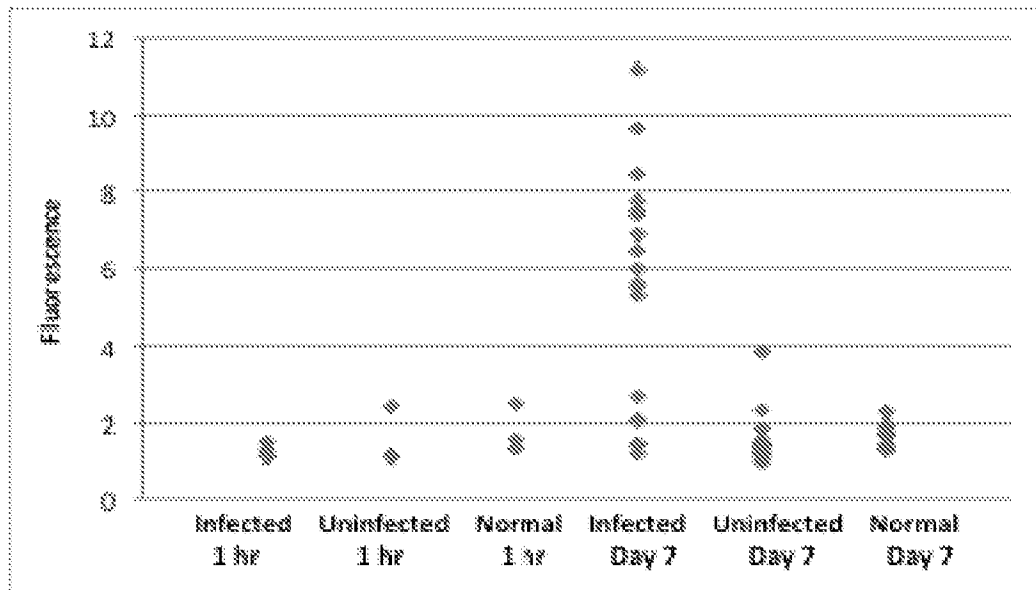


Fig. 3

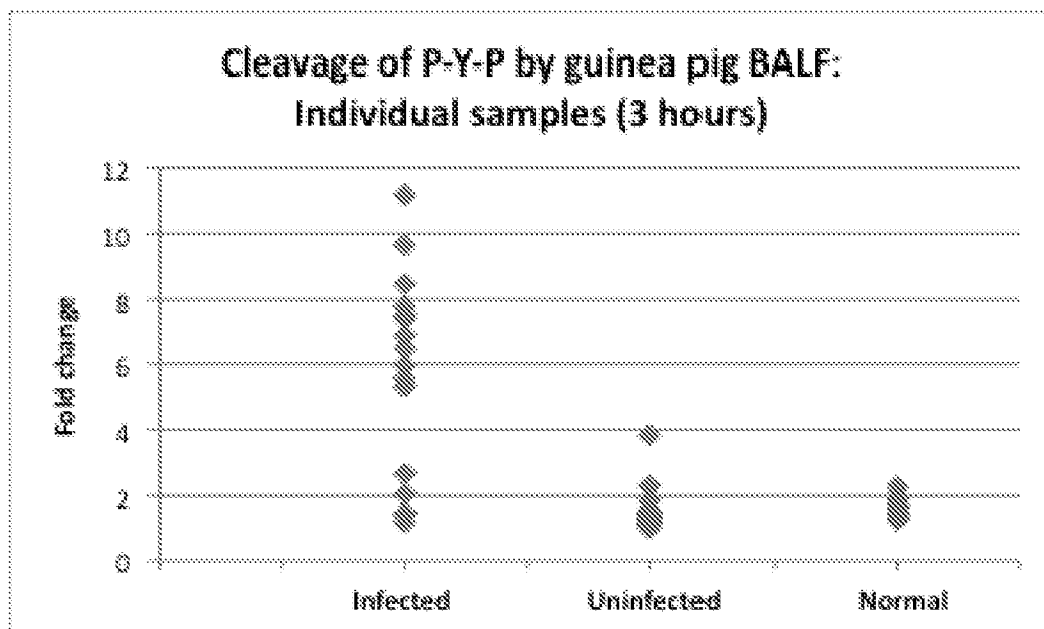


Fig. 4

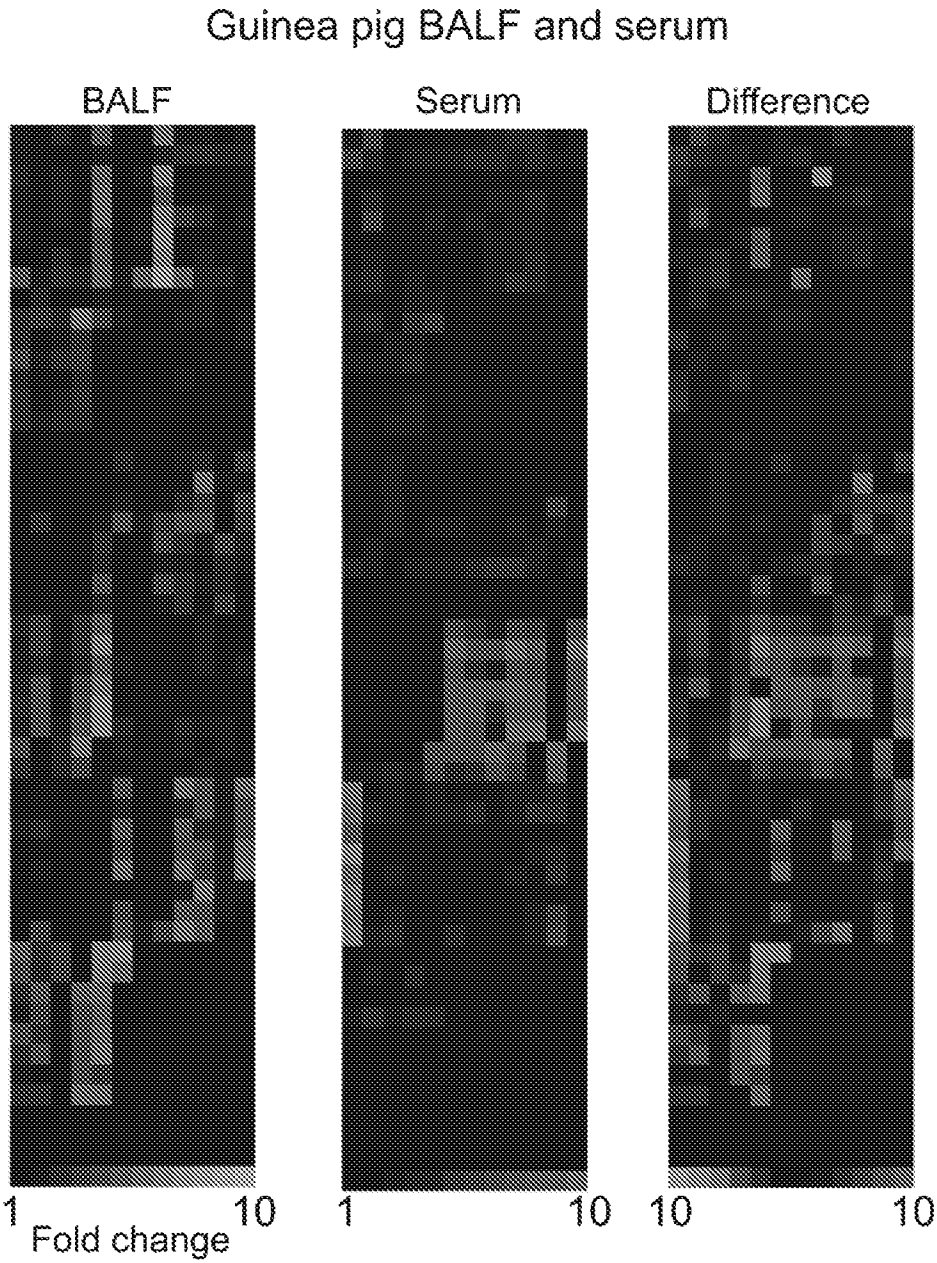


Fig. 5

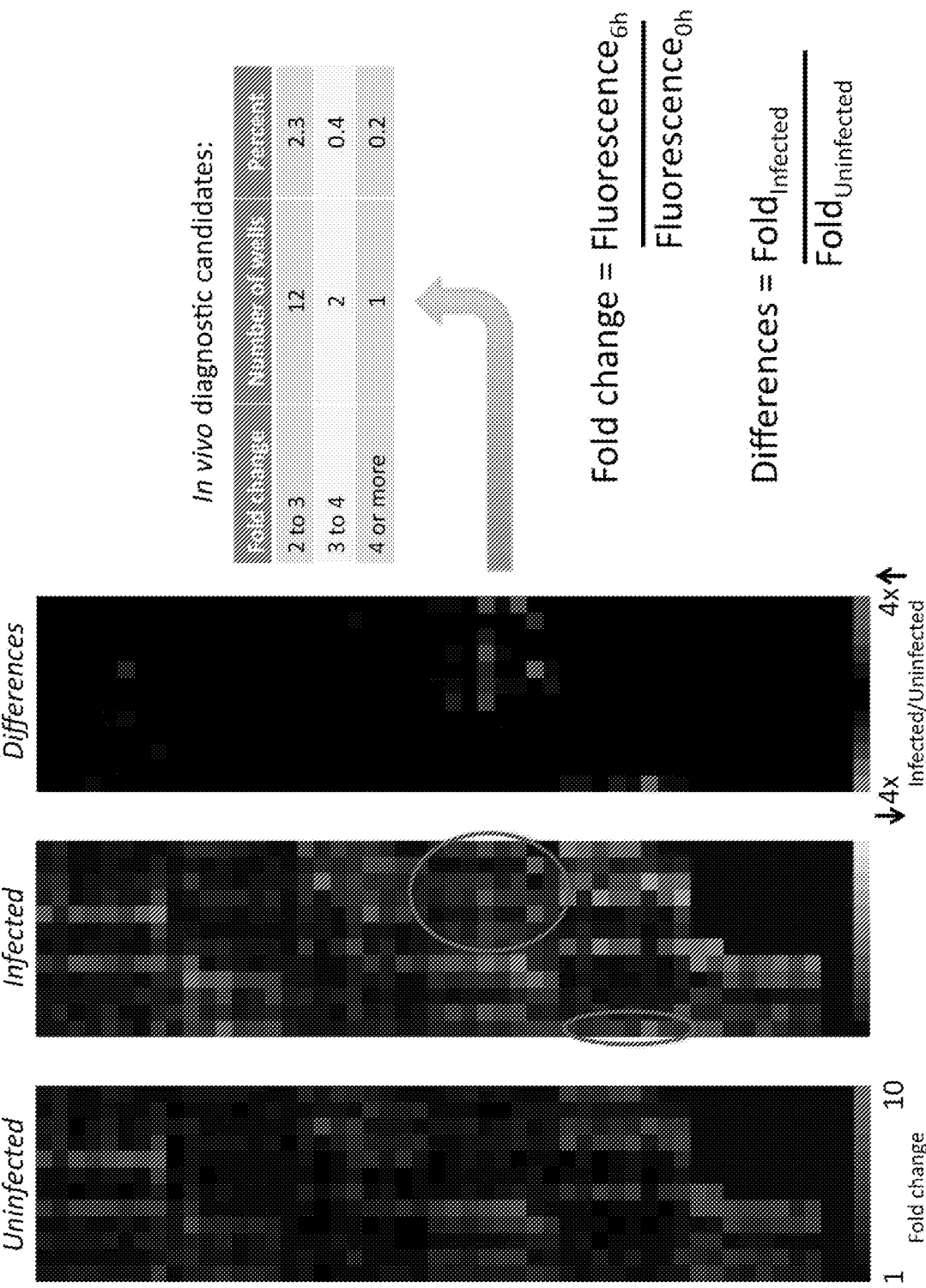


Fig. 6.

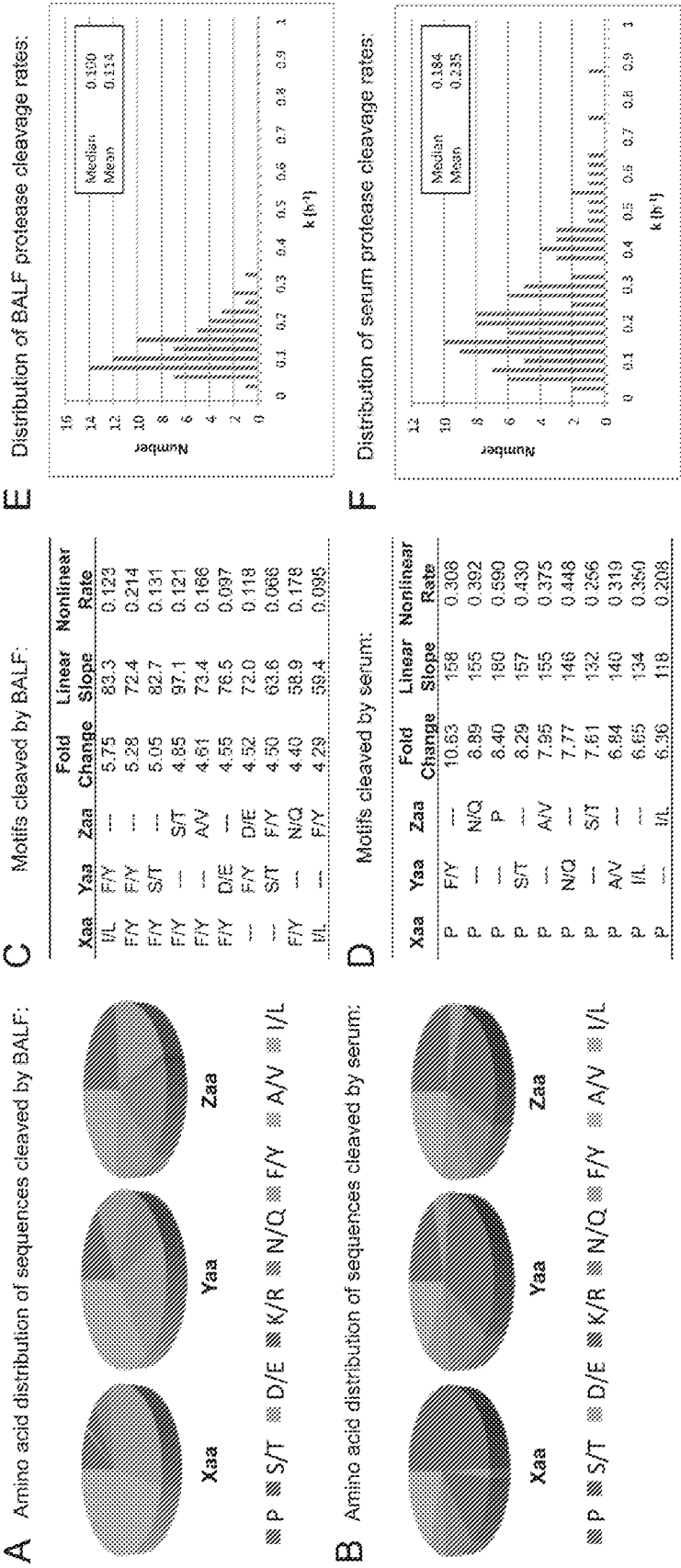


Fig. 7