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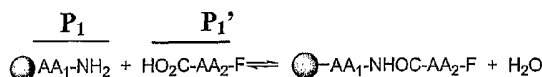
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(54) Title: **PROTEASE ASSAY**



(57) **Abstract:** The present invention relates to a method for detecting or determining the activity of a protease or putative protease. The present invention also provides products and reagents suitable for use in the method, as well as uses thereof.



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PROTEASE ASSAY

Field of the Invention

The present invention relates to a method for detecting or determining the activity of a protease or putative protease. The present invention also provides products and reagents suitable for use in the method, as well as uses thereof.

Background to the Invention

An estimated 2-3% of the mammalian proteome consists of proteases: enzymes that hydrolyze proteins and peptides. Many of these enzymes are involved in essential physiological functions such as immunological defense and cell differentiation by selective proteolysis of cell surface receptors.¹ Other proteases are involved in disease states, such as HIV,^{1c} Alzheimer's disease,^{1d} Hepatitis C,^{1e} Candida infections,^{1f} and pancreatitis.^{1g} Many proteases have also been used successfully in organic synthesis.²

Proteases differ from one another in a distinct preference for certain amino acids at positions on either side of the amide cleavage site. The function of a particular protease is primarily determined by the amino acids in the P₁ and P₁' positions: those directly adjacent to the amide cleavage site. Given the large number of proteases and their importance in both synthetic and medicinal chemistries high-throughput screening methods for protease specificity are required.

Over the past years several methods have been developed to determine the specificity of proteases. Phage display libraries, for example, permit the determination of protease specificity by exposing vast numbers of recombinant peptides to a given protease.³ Meldal and co-workers developed screening methods on large combinatorial peptide (or peptide mimic) libraries with fluorogenic labels. Both the synthesis and the biological screening of these libraries could be carried out directly on solid PEGA (poly acrylamide poly ethylene

glycol co-polymer) supports that are compatible with enzyme activity.⁴ Recently, the first successful peptide chip was described where the members of a peptide library were directly arrayed onto glass slides. However, this method is so far restricted to analysis of specificity for amino acids positioned on the carboxylic side of the cleaved peptide bond.⁵

Nevertheless, some of the above processes suffer from a number of disadvantages. For example, conventional library approaches are demanding in terms of synthesis. Moreover, most existing methods only distinguish between hits and non-hits in a relatively qualitative manner. Additionally methods based on longer peptides require sequencing of peptides on "hit" beads.

It is amongst the objects of the present invention to obviate and/or mitigate at least one of the aforementioned disadvantages and/or to provide a simpler method which may be more suited to high-throughput applications.

Summary of the Invention

The present invention is based in part on a novel conceptually different method for the profiling of the primary (P_1 and P_1') specificities of proteases. Some of the present inventors recently reported the observation that the peptide hydrolysis equilibrium can be shifted toward peptide synthesis when the carboxylic acid terminus of the P_1 amino acid is attached to a solid PEGA support⁶. Nevertheless there was no suggestion that this could be used to develop an assay for detecting and/or determining activities of proteases with hitherto unknown activities.

Thus, in a first aspect there is provided a method for detecting and/or determining protease activity, comprising the steps of:

a) providing a series of different amino acids immobilised on a substrate wherein the alpha amino group of each amino acid is capable of reacting with an alpha

carboxyl group of a labelled amino acid in order to form a peptide bond;

b) forming a series of reaction mixtures by contacting the labelled amino acid with said series of different amino acids;

c) providing a sample comprising a protease or putative protease to said reaction mixtures and subjecting the reaction mixtures to conditions suitable to allow peptide bond synthesis to occur between any of said different amino acids and said labelled amino acid;

d) substantially removing any unreacted labelled amino acid from said reaction mixtures; and

e) observing whether or not a peptide bond has been formed between any of said bound amino acids and said labelled amino acid.

Typically the series of different amino acids corresponds to a series comprising all 20 naturally occurring amino acids, namely: Alanine (A), Cysteine (C), Aspartic Acid (D), Glutamic Acid (E), Phenylalanine (F), Glycine (G), Histidine (H), Isoleucine (I), Lysine (K), Leucine (L), Methionine (M), Asparagine (N), Proline (P), Glutamine (Q), Arginine (R), Serine (S), Threonine (T), Valine (V), Tryptophan (W), Tyrosine (Y). In this manner it is possible to detect and/or determine the primary specificity of a protease. That is, it is possible to detect and/or determine which amide bond or bonds, the test protease has specificity for, from the 20 amide bonds tested. The present invention provides a method of being able to quantify the amount of label and in turn determine the relative preference for different substrate bond amino acids. The protease may be known, in the sense that it is known to function as a protease, but its specificity is unknown, or a sample may be tested in order to ascertain if any protease is present therein.

Additionally/alternatively non-naturally occurring or unusual amino acids may be used in the method, as part of the series of different amino acids and/or as the labelled amino acid. In this manner, it is possible to detect and/or determine whether or not a protease or

putative protease has specificity for forming an amine bond between a naturally occurring amino acid and a non-natural amino acid, and/or a non-natural amino acid and another non-natural amino acid. Examples of non-natural amino acids include, 2-Aminoadipic acid, 3-Aminoadipic acid, beta-Alanine, beta-Aminopropionic acid, 2-Aminobutyric acid, 4-Aminobutyric acid, piperidinic acid, 6-Aminocaproic acid, 2-Aminoheptanoic acid, 2-Aminoisobutyric acid, 3-Aminoisobutyric acid, 2-Aminopimelic acid, 2,4-Diaminobutyric acid, Desmosine, 2,2'-Diaminopimelic acid, 2,3-Diaminopropionic acid, N-Ethylglycine, N-Ethylasparagine, Hydroxylysine, allo-Hydroxylysine, 3-Hydroxyproline, 4-Hydroxyproline, Isodesmosine, allo-Isoleucine, N-Methylglycine, sarcosine, N-Methylisoleucine, 6-N-Methyllysine, N-Methylvaline, Norvaline, Norleucine, Ornithine.

The substrate to which the series of different amino acids are immobilised, may be any suitable substrate, such as a plastics material e.g. polyethylene glycol acrylamide (PEGA), polypropylene, polystyrene, silica (glass) or metal substrates such as gold. Conveniently, the series of immobilised amino acids are provided as an array. That is the immobilised amino acids are provided in distinct spaced apart locations. For example if all 20 amino acids are immobilised, this may be provided as a 5 x 4 array.

In one embodiment the immobilised amino acids may be bound to a resin or bead, such as PEGA which is then added to a receptacle such as a well of, for example, a microtitre plate. A 96-well microtitre plate is suitable, although plates with less or greater numbers of wells could also be utilised. The skilled addressee can easily envisage how this could therefore be adapted to an automated and/or semi-automated process. In another embodiment the series of immobilised amino acids may be provided as an array on a single substrate, for example as a micro-array or micro-chip as known in the art. In this manner the array can be extremely small e.g. microns to millimetres² in area. Moreover, miniature, for

example, hand-held or table-top devices are and have been developed for processing and reading of such micro-arrays

Immobilisation of the amino acids may be by any suitable means, but is typically by bond formation, such as an ionic or covalent bond. Linking may be direct to the substrate, or a linker may be provided to facilitate bonding between the amino acid and the substrate. Suitable linkers include safety catch linkers, Merrifield linker and Wang linker. The substrate to which the amino acid is to be immobilised may, for example, comprise a functional group or groups suitable for reacting with the amino acid, or the substrate can be modified so as to enable immobilisation of the amino acid thereto. Conveniently the substrate may comprise or be modified to comprise a reactive group such as an amino group which can react to form a bond with for example the alpha carboxyl group of the amino acid to be immobilised. For example glass may be modified to comprise amino terminated silanes e.g. amino propyl silane, to which an amino acid may be bound.

Forming the series of reaction mixtures may be carried out by contacting a solution comprising the labelled amino acid with the immobilised amino acids. In an embodiment which utilises receptacles, such as wells, for holding the substrate bound amino acids, the labelled amino acid may simply be added also to the receptacle. In an embodiment employing for example a micro-array, the labelled amino acid may be added to the surface thereof, or for example, the micro-array could have microfluidic channels for transporting the labelled amino acid to the series of bound amino acids.

Again, the sample comprising the protease or putative protease may be, for example, added to the well comprising the bound amino acid and labelled amino acid, or added to the surface of micro-array by pipetting or using microfluidic means known in the art. Additional agents as may be required for protease action may also be added at this time. Typically the reaction mixtures with added protease or putative protease are left for a period

of time e.g. 1 - 24 hours, typically 2 - 12 hours and at a suitable temperature e.g. 4°C - 60°C, typically 15°C - 45°C e.g. 37°C to allow the protease to carry out a peptide bond synthesis reaction. Naturally it is understood that any given protease is likely to be quite specific in that only a small number of peptide synthesis reactions may be successful and to certain degrees. The methods of the present invention, as opposed to some of the prior art, can be seen as quantitative or at least semi-quantitative as it is possible to observe degrees of specificity which can be quantified, such that the methods provide more than just a yes/no answer.

The peptide bond making/breaking process is actually an equilibrium reaction and to drive the reaction in the direction of synthesis an excess of Fmoc-amino acid and a small volume of buffer solution is required. Typically an excess of 4-10-fold of amino acid is generally required for complete coupling. There are several reasons why a shift in equilibrium toward synthesis can be expected when the amine substrate is linked to a solid support. One widely recognized advantage of solid-phase synthesis is that large excesses of substrates can be used to help drive reactions to completion. A second contribution is expected from suppressed ionization of the solid supported amine due to the overall positive charge of the resin. A final effect may result from the improved solvation of hydrophobic acyl donors in PEGA resin when compared to aqueous environment. This increase will result in a local higher concentration of acyl donor near the site of catalysis.

It is important to remove any unreacted labelled amino acid from the reaction mixtures, otherwise it would not be possible to discern labelled unbound amino acid from labelled bound amino acid (i.e. a labelled amino acid which has formed a peptide bond). Typically removal of unbound labelled amino acid is carried out by a washing step or series of steps. Detection of bound labelled amino acid can then be carried out.

Conveniently labelling of the amino acid is carried out by using a fluorescent label which can easily be detected by conventional means such as by fluorescent plate readers known in the art. A particularly suitable label is the Fmoc protection group which may be bound to the alpha amino group of the amino acid in order to provide the label and moreover, stop the amino acid from being able to react further. Other labels may also be suitable, such as radiolabels and antibodies which may be detected by techniques such as ELISA.

Aptamers⁹ may also be used as a means of either labelling antibodies or for direct labelling of amino acids bound to the solid substrate. Advantageously the aptamers are labelled by some means, for example by the use of radionucleotides.

The methods described herein may find application in a number of fields. For example, the methods may be used to assist drug delivery through target identification. A significant number (estimated to be 2-3%) of the proteins encoded by the human genome are proteases. Proteases therefore present one of the most important class of targets for the pharmaceutical industry. A medicinal chemistry programme aimed at developing inhibitors of a target protease would benefit from trying to establish the substrate specificity of the enzyme. Such peptide recognition sequence could then be used as useful leads for further drug development. As a consequence, screening for the substrate specificity of an unknown protease is an important first step.

Moreover, the methods could be used for high-throughput screening of compound libraries. There is a continued demand for robust cheap high-throughput systems for screening compound libraries as potential inhibitors of a protease.

In diagnostic Kits the methods may also be employed where a protease would be a disease marker.

Additionally, biocatalysts are important tools for the fine chemical industry. Proteases are used e.g. for the production of amino acids and derivatives (the

sweetener aspartame is produced using a protease catalysed synthetic step), and the partial hydrolysis of proteins for applications in food or feed, and as non-specific hydrolyses in detergents.

There is a need to provide novel biocatalysts for the fine chemicals industry, using as one method of catalyst development the method of directed evolution. Here, a large number of novel enzymes can be generated by random mutagenesis techniques. The substrate specificity of such novel enzymes needs to be established and high-throughput microarray methods such as the ones described herein will be useful tools.

Moreover, proteases are also used in bulk in "biological" washing powders. Directed evolution can and has been used to find enzymes with optimised activities.

In a further aspect the present invention provides an amino acid array for use in detecting and/or determining protease activity, the array comprising a plurality of naturally occurring amino acids immobilised to a substrate or substrates wherein each amino acid is immobilised such that the alpha amino group of each amino acid is capable of reacting with an alpha carboxyl group of an unbound amino acid for forming a peptide bound.

Preferably the array comprises all 20 natural amino acids.

Preferably the array is provided in the form of a micro-array comprising a single substrate to which is bound said plurality of amino acids.

In a further aspect the present invention provides a kit for use in detecting and/or determining protease activity, the kit comprising an amino acid array comprising a plurality of naturally occurring amino acids immobilised to a substrate or substrates wherein each amino acid is immobilised such that the alpha amino group of each amino acid is capable of reacting with an alpha carboxyl group of an unbound amino acid for forming a peptide bound and a source of free labelled amino acid for reacting with any of said immobilised amino acids,

Typically the free amino acid is labelled with a fluorescent group, such as F_{moc} and this may occur on the alpha amino group of the free amino acid, leaving the alpha carboxyl group free to react with the alpha amino group of the immobilised amino acid.

The present invention exploits the reversibility of the peptide synthesis/hydrolysis reaction: instead of looking at the cleavage of peptides, the reverse reaction, i.e. the specific attachment of labelled amino acids is used. It can be hypothesised that the primary specificity for both the synthesis and hydrolysis reactions are the same for each amino acid couple since for the reactions in both directions the same transition states are involved.

The present invention will now be described by way of Example and with reference to the Figures which show:

Figure 1 shows the peptide synthesis/hydrolysis equilibrium on solid support (AA are amino acids and F' is a fluorescent group);

Figure 2 shows a schematic of an experimental set up with 20 different amino acids as immobilised on PEGA₁₉₀₀ beads and how on addition of an F_{moc} labelled amino acid and action by a protease, this can lead to a labelled product;

Figure 3 and 4 show primary thermolysin specificity as determined by solid phase micro assay. P₁ amino acids were F_{moc} labelled while P₁' amino acids were linked to the solid phase. Error bars represent standard deviation obtained from a number of experiments; and

Figure 5 shows a schematic of binding various amino acids to silica which has been modified by the addition of propyl silane.

Examples Section

Overview

In a 96 well filter plate (Captiva by Ansys Technologies) in separate wells the twenty naturally occurring L-Fmoc amino acids (P₁') are linked to the PEGA₁₉₀₀-NH₂ resin (Polymer Laboratories) using

conventional peptide coupling chemistries. After deprotection, the 20 different polymer bound amino acids are then exposed to a protease in the presence of a second, soluble Fmoc-protected amino acid (P_1) to the first the resin bound amino acids (Scheme 1, Figure 1). Peptide synthesis will only occur if the protease primary specificity is compatible with amino acid couple in each well. Successful couplings can be detected by taking advantage of the fluorescence of the Fmoc group, detected in a plate reader. Using all twenty soluble P_1 ' amino acids, the complete protease primary specificity for each P_1P_1' combination can be obtained.

Four reactions are performed for each P_1 ' amino acid. A fully deprotected resin-bound amino acid P_1 ' is measured to get background values. Secondly, a side-chain deprotected, but Fmoc protected resin-bound amino acid P_1 is measured to determine the loading levels. Third and fourth are the actual enzymatic reactions with the second (P_1) amino acid in duplicate. So for one assay (one P_1 amino acid, 20 P_1 ' amino acids) 80 wells per plate are used.

Step 1: Preparing the resin and the amino acid solutions.

Water used in all steps is of protein synthesis quality (Millipore).

75 mg of wet resin (typically neutral PEGA₁₉₀₀) was used per reaction (well). This wet resin contained 9.5% dry weight and the loading of functional amino groups was 0.2 mmol/gram dry weight. Each reaction well therefore contained 1.4×10^{-3} mmol of amino-functionality.

a) Preparation of the Resin:

(For 80 reactions per plate). 6.375 g wet resin was added to 85 ml Millipore water. The beads were transferred by pipette in 5x200 μ l aliquots to each well. Water was drained off using a vacuum manifold and the resin washed 3x with DMF_{anhydrous} (N,N-Dimethylformamide anhydrous).

b) Addition of Amino acids:

Ten equivalents of fully protected amino acids per reaction were used. 0.06 mmol of each of the 20 amino acids P_1 were added to each well of a 96 well plate add 1ml $DMF_{anhydrous}$ was added to each these wells.

c) Preparation of HOBt/DIC solution:

Hydroxybenzotriazole (HOBt) and Diisopropylcarbodiimide (DIC) were used to chemically couple the first amino acid onto the resin. For a stock solution 190mg of HOBt and 220 microliters of DIC were mixed with 30 ml of DMF.

Step 2: Chemical coupling of the first amino acid.

In each well 500 μ l of amino acid solution (1b) and 600 μ l HOBt/DIC solution (1c) was left to react for half an hour before using it in the coupling step. 550 μ l of this solution was then added to each well containing resin (1a) together with 350 μ l DMF. The mixture was left to react for three hours. The resin was then washed with the following: 3x $DMF_{anhydrous}$, 3x $DMF_{anhydrous}/MeOH$ (1:1), 3x THF (Tetrahydrofuran), 3x DCM (Dichloromethane), 3x MeOH.

Step 3: De-protection of the side chains:

To be able to perform the enzymatic synthesis on the P_1' amino acids all the side chain protection groups have to be removed.

Thus 1.0 ml TFA/TIS/ H_2O (95:2.5:2.5) was added to all of the wells containing resin-bound amino acids with acid-labile side chain protection groups and the mixture was left to react for three to four hours.

In addition, to the wells containing Tryptophan as the P_1' amino acid, 1 ml H_2O was added, incubated for a few minutes, the supernatant removed and the treatment repeated. These wells were then again washed with 4x H_2O .

Finally, all wells were washed with 4x DMF_{anhydrous}.

Step 4: Removal of the N-terminal Fmoc protection group:

The next step was to remove the Fmoc protection group. To all wells except those that retained Fmoc protection as a control was added 1.0 ml 20% Piperidine in DMF_{anhydrous} and the mixture was left for two hours to complete the deprotection.

All wells were then washed with 3x DMF_{anhydrous}, 3x DMF_{anhydrous} /MeOH (1:1), 3x THF, 3x DCM, 3x DMF_{anhydrous}

Step 5: Protease screen.

The P₁ amino acids to be coupled onto the P₁' amino acids need to have a N-terminal Fmoc protection group, but should not have side chain protection groups. Because of the subsequent fluorescence measurements, care was taken that no free Fmoc amino acids remain in the resin. Therefore extensive washing is needed, with water as the last step to remove all solvents before fluorescence measurements.

To each well a solution of 1 ml potassium phosphate buffer 0.1 M pH 8.0 , 5 eq. of the P₁ Fmoc amino acid (no side chain protection groups) and 2 mg protease was added and the mixture incubated overnight.

The wells were then washed with 5X MeCN/H₂O (1:1) with 0.1%TFA, 5X DMF_{anhydrous}, 3X DMF_{anhydrous} /MeOH (1:1), 3X MeOH, 5X MeCN/H₂O (1:1), 3X MeOH, 5X H₂O.

Step 6: Fluorescence measurements:

To each well 900 µl H₂O was added and the mixture left to soak for 1 hour. The beads suspension were homogenised several times using a pipette (cut off the end of the tips) immediately before transferring the beads to the sample plates.

All reaction samples were distributed over four wells of a transparent 96 well plate used in the plate

reader in aliquots of 200 μ l /well. Fluorescence measurements were conducted using a Spectramax Gemini XS at Excitation wavelength =299 nm and Emission wavelength =315 nm. An average of 25 readings/well was performed to reduce errors in the measurements.

The difference between the emission intensity of the background (fully de-protected P_1' amino acid on the resin) and that of the same sample after treatment with protease was used as a measure for the synthesis per P_1' - P_1 combination.

Figures 3 and 4 shows results using thermolysin as an example protease. This enzyme is a member of the M4 peptidase family (metalo-proteases) that includes several enzymes that play crucial roles *in vivo*⁷ and it also well known for its industrial use in the synthesis of the low calorie sweetener aspartame⁸.

The primary specificity of thermolysin has been well documented. See for example Fluka catalogue. The preferred amino acids are known to be the large, hydrophobic amino acids in P_1' position while the enzyme is known to be non-specific in the P_1 position. The reported specificity for Leu, Ile and Phe is indeed observed for the four P_1 amino acids studied here. It was found that by systematically reducing the side chain length there was an overall decrease in specificity in the order Ile/Leu, Val, Ala/Gly where side chains get less bulky and less hydrophobic. Tryptophan is known to be too bulky for the enzymatic reaction to occur, and is not accepted by thermolysin as was previously observed.

Figures 3 and 4 also reveal that the nature of P_1 significantly effects the selectivity for P_1' in thermolysin. It appears that a large hydrophobic phenyl group in P_1 restricts P_1' to large hydrophobic amino acids (mainly Ile, Leu and to a lesser extent Val, Met and Phe) while for smaller Gly the substrate specificity is more relaxed an a wider range of substrates are accepted.

In addition to PEGA substrates, glass has also been modified so as to allow immobilisation of amino acids. Glass microscope slides were first treated with amino propyl silane to functionalise the surfaces with amino groups 1. See Figure 5. XPS analysis confirmed the presence of s1 carbon and nitrogen atoms on the glass surfaces. Next, the glass bound amines were acylated with four different Fmoc protected amino acids. The amino acids that were chosen had increasingly hydrophobic side chains: Glycine, Alanine, Leucine and Phenylalanine to create surfaces 2a-d. Next, the Fmoc protecting groups were removed to give 3a-d and the glass bound amino acids were subsequently acylated with Fmoc-phenylalanine to give surface bound di-peptides 4a-d.

The methods presented herein are based on reaction thermodynamics indicating that there is no fundamental reason why the screen should not be successful for other proteases. Literature reports leave no doubt that many proteases are active on peptides that are linked to PEGA beads. If any proteases will not tolerate the Fmoc group linked to the N terminus of the P₁ amino acid, this problem can be overcome by introducing spacer groups or using different fluorophores.

In summary, by taking advantage of enzymatic amino acid coupling on a solid support primary screening of protease specificity is greatly simplified. It was shown that the primary specificity of thermolysin could be readily determined through the coupling of fluorescent amino acids.

The suggested approach has a significant advantage over existing methods in that it involves the immobilisation of just 20 amino acids on solid beads. In addition, the method inherently requires immobilisation onto a solid support to allow for peptide synthesis to occur. Hence, the approach is promising for use in protease primary profiling on solid phase bio-chips.

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Claims:

1. A method for detecting and/or determining protease activity, comprising the steps of:
 - a) providing a series of different amino acids immobilised on a substrate wherein the alpha amino group of each amino acid is capable of reacting with an alpha carboxyl group of a labelled amino acid in order to form a peptide bond;
 - b) forming a series of reaction mixtures by contacting the labelled amino acid with said series of different amino acids;
 - c) providing a sample comprising a protease or putative protease to said reaction mixtures and subjecting the reaction mixtures to conditions suitable to allow peptide bond synthesis to occur between any of said different amino acids and said labelled amino acid;
 - d) substantially removing any unreacted labelled amino acid from said reaction mixtures; and
 - e) observing whether or not a peptide bond has been formed between any of said bound amino acids and said labelled amino acid.
2. The method of claim 1 wherein the series of different amino acids corresponds to a series comprising all 20 naturally occurring amino acids.
3. The method of claim 1 or 2 wherein the series of different amino acids and/or the labelled amino acids further comprise non-naturally occurring or unusual amino acids.
4. The method of any preceding claim wherein the substrate to which the series of different amino acids are immobilised, is a polymeric material.

5. The method of claims 1-3 wherein the substrate to which the series of different amino acids are immobilised is silica (glass) or a metal substrate.
6. The method of any preceding claim wherein the series of amino acids are immobilised directly to the substrate.
7. The method of claims 1-5 wherein the substrate to which the amino acid is to be immobilised comprises a functional group or groups suitable for reacting with the amino acid.
8. The method of claims 1-5 wherein the substrate is modified so as to enable immobilisation of the amino acid thereto.
9. The method of claims 7 or 8 wherein the substrate comprises or is modified to comprise, a reactive group which can react to form a bond with the amino acid to be immobilised.
10. The method of claims 7-9 wherein the series of amino acids are immobilised to the substrate via a linker.
11. The method of claim 10 wherein the linker is selected from one of the following; safety catch linkers, Merrifield linker and Wang linker.
12. The method of any preceding claim wherein the series of immobilised amino acids are provided as an array.
13. The method of claim 12 wherein the series of immobilised amino acids are immobilised on a single substrate as a micro-array or micro-chip.
14. The method of any preceding claim wherein the substrate has microfluidic channels for transporting the labelled amino acid to the series of bound amino acids.

15. The method of claims 1-5 wherein the immobilised amino acids are bound to a resin or bead, such as poly acrylamide poly ethylene glycol co-polymer (PEGA).
16. The method of any preceding claim wherein the formation of a series of reaction mixtures may be carried out by contacting a solution comprising the labelled amino acid with the immobilised amino acids.
17. The method of any preceding claim wherein the labelled amino acid is added to a receptacle for holding the substrate bound amino acids.
18. The method of any preceding claim wherein the sample comprising the protease or putative protease is added to the immobilised amino acid and labelled amino acid by pipetting or using microfluidic means.
19. The method of any preceding claim wherein the reaction mixtures with added protease or putative protease are left for 1 - 24 hours at 4⁰C - 60⁰C to allow the protease to carry out a peptide bond synthesis reaction.
20. The method of any preceding claim wherein removal of unbound labelled amino acid is carried out by a washing step or series of steps.
21. The method of any preceding claim wherein the labelled amino acid is labelled with a fluorescent label.
22. The method of claim 23 wherein the fluorescent label is the F_{moc} protection group.
23. The method of claims 1-20 wherein the labelled amino acid is labelled with a radioactive label.

24. The method of claims 1-20 wherein the labelled amino acid is labelled with an antibody.
25. The method of claims 1-20 wherein the labelled amino acid is labelled with an aptamer.
26. The method of claim 24 wherein the antibody is labelled with an aptamer.
27. An amino acid array for use in detecting and/or determining protease activity, the array comprising;
a plurality of naturally and/or non-naturally occurring amino acids immobilised to a substrate or substrates wherein each amino acid is immobilised such that the alpha amino group of each amino acid is capable of reacting with an alpha carboxyl group of an unbound amino acid for forming a peptide bound.
28. The array of claim 25 wherein the array further comprises all 20 natural amino acids.
29. The array of claim 26 wherein the array is provided in the form of a micro-array further comprising a single substrate to which is bound said plurality of amino acids.
30. A kit for use in detecting and/or determining protease activity, the kit comprising an amino acid array comprising;
a plurality of naturally occurring amino acids immobilised to a substrate or substrates wherein each amino acid is immobilised such that the alpha amino group of each amino acid is capable of reacting with an alpha carboxyl group of an unbound amino acid for forming a peptide bond; and
a source of free labelled amino acid for reacting with any of said immobilised amino acids.

31. The kit of claim 28 wherein the free amino acid is labelled with a fluorescent group.

Figure 1

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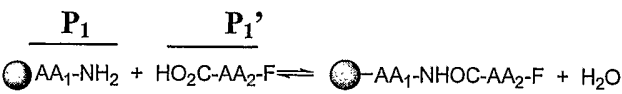


Figure 2

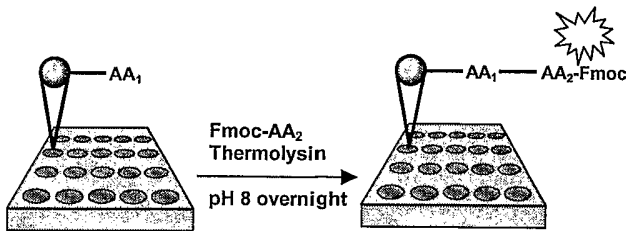
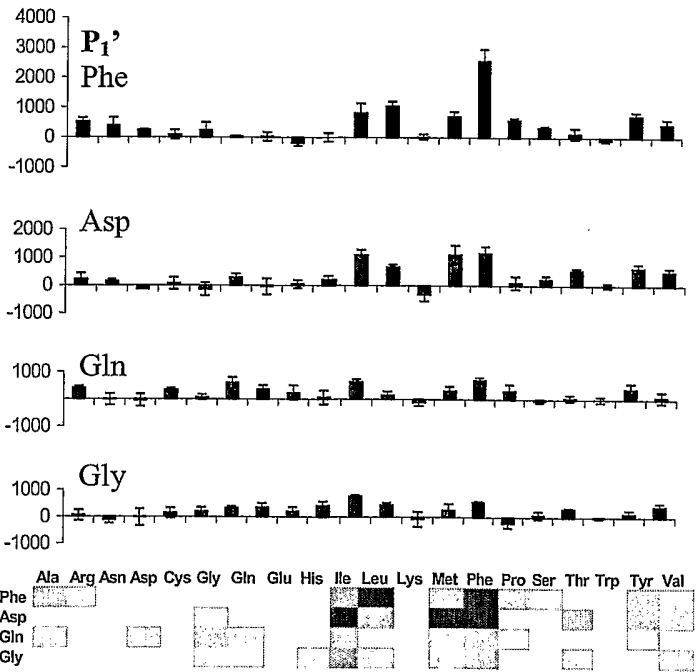


Figure 3



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Figure 4

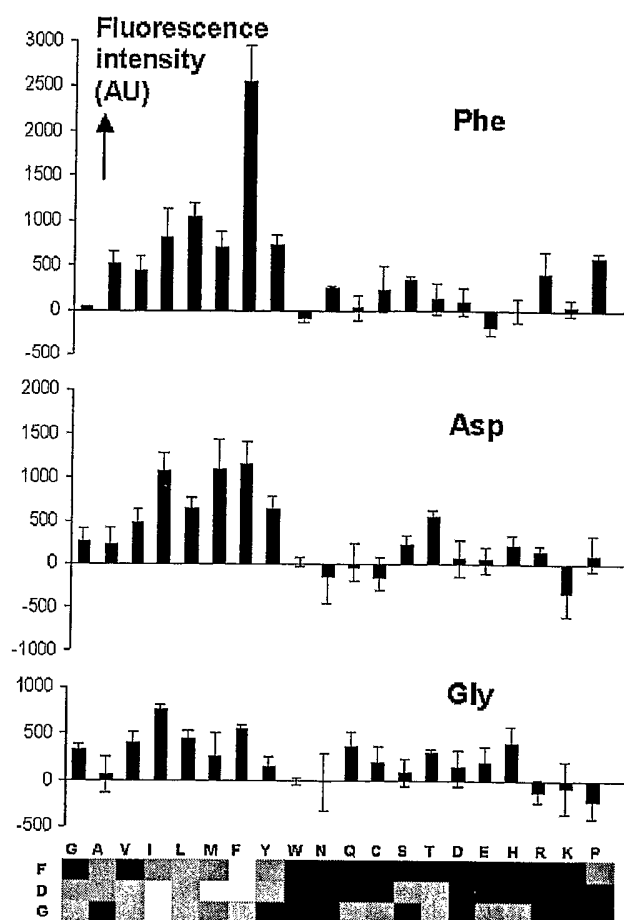
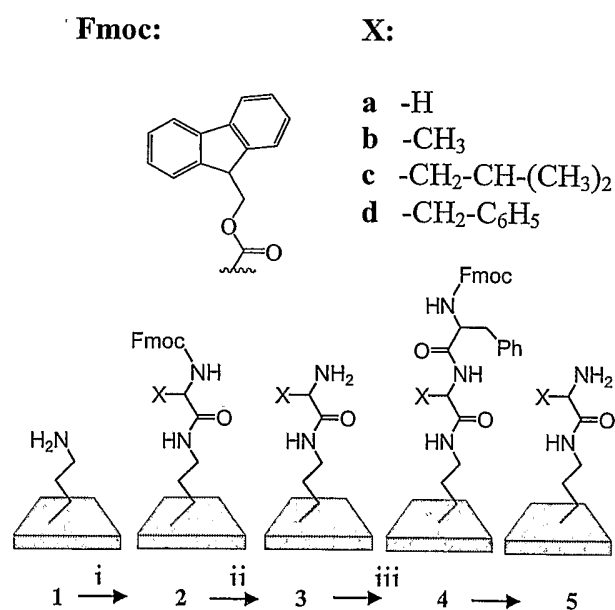


Figure 5



INTERNATIONAL SEARCH REPORT

tional Application No
PC1/GB2004/003299

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12Q1/37		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/53625 A (MONTGOMERY DONALD D ; ROSSI FRANCIS M (US); COMBIMATRIX CORP (US)) 14 September 2000 (2000-09-14) p.8, paragraph concerning figure 9; page 23, line 19 - line 31; figure 9	27,29
A	SALISBURY C M ET AL: "Peptide microarrays for the determination of protease substrate specificity" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 124, no. 50, 18 December 2002 (2002-12-18), pages 14868-14870, XP002277823 ISSN: 0002-7863 the whole document	1-31
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.		
° Special categories of cited documents :		
A document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *8* document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
24 November 2004	06/12/2004	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Jacques, P

INTERNATIONAL SEARCH REPORT

ational Application No
PCT/GB2004/003299

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ULIJN REIN V ET AL: "Protease-catalyzed peptide synthesis on solid support" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 124, no. 37, 18 September 2002 (2002-09-18), pages 10988-10989, XP002307319 ISSN: 0002-7863 the whole document -----	1-31
A	ULIJN REIN V ET AL: "Understanding protease catalysed solid phase peptide synthesis." ORGANIC & BIOMOLECULAR CHEMISTRY. 21 APR 2003, vol. 1, no. 8, 21 April 2003 (2003-04-21), pages 1277-1281, XP002307320 ISSN: 1477-0520 the whole document -----	1-31
P,X	R.H.P. DOEZÉ, B.A. MALTMAN, C.L. EGAN, R.V. ULIJN, S.L. FLITSCH: "Profilig primary protease specificity by peptide synthesis on a solid support" ANGEWANDTE CHEMIE, vol. 116, 14 June 2004 (2004-06-14), pages 3200-3203, XP002307314 the whole document -----	1-31

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