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(71) Applicant(s):
Mologic Ltd
(Incorporated in the United Kingdom)
Colworth Science Park, SHARNBROOK,
Beds, MK44 1LQ, United Kingdom

(72) Inventor(s):
Mark James Davis
Paul James Davis
Mark Burnapp

(74) Agent and/or Address for Service:
Lloyd Wise
Commonwealth House,
1-19 New Oxford Street, LONDON,
WC1A 1LW, United Kingdom

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G1B BCB

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INT CL **C12Q, G01N**
Other: **WPI, EPODOC, BIOSIS, STN CAPLUS**

(54) Abstract Title: **A protease detection product**

(57) A protease detection product (1) for detecting a protease enzyme in a sample. The protease detection product (1) comprises a medium (2) providing a liquid flow path; a labelled component (6) located on the liquid flow path; a capture component (12) located downstream of the labelled component (6) and immobilisation means (8) for immobilising the intact labelled component. The labelled component (7) comprises a base element (8) connected to releasable element via a protease-sensitive linker peptide (9). The releasable element comprises a label (10). The capture component (12) is capable of binding the releasable element.

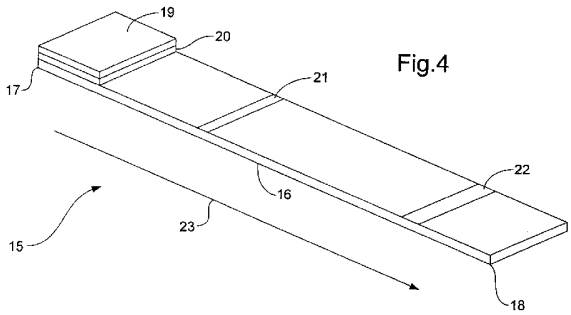


Fig.4

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Fig.1

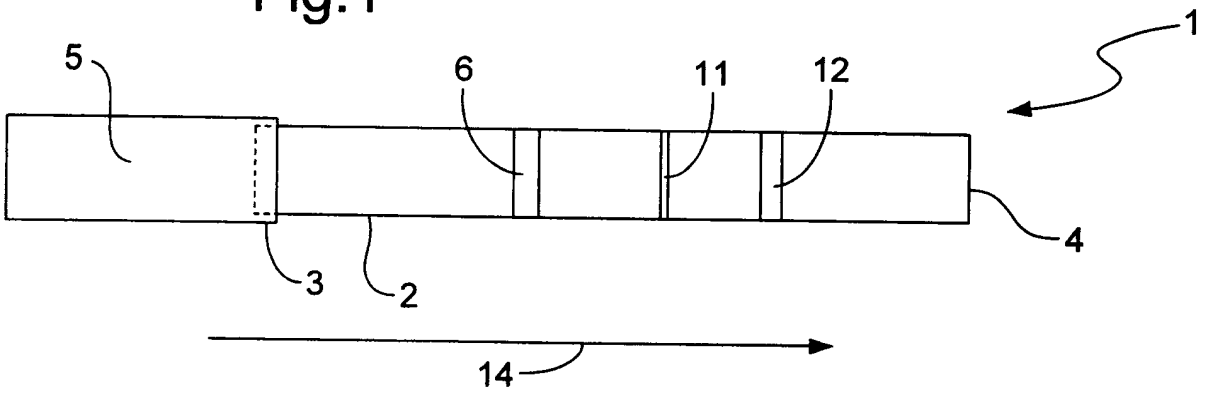


Fig.2

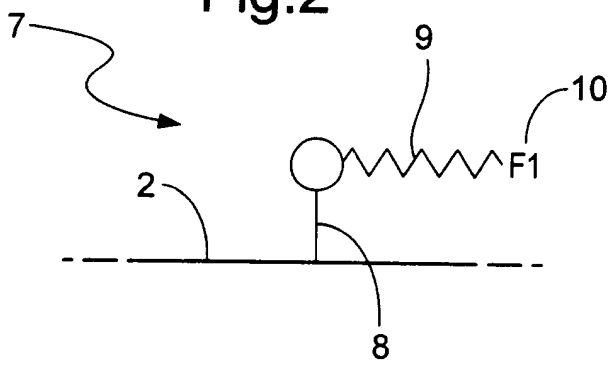


Fig.3

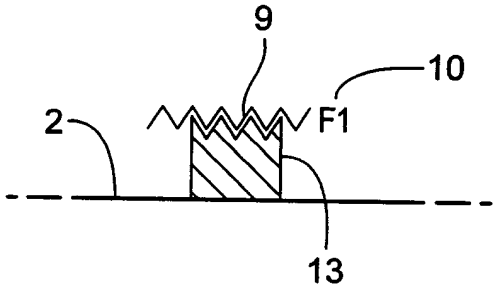
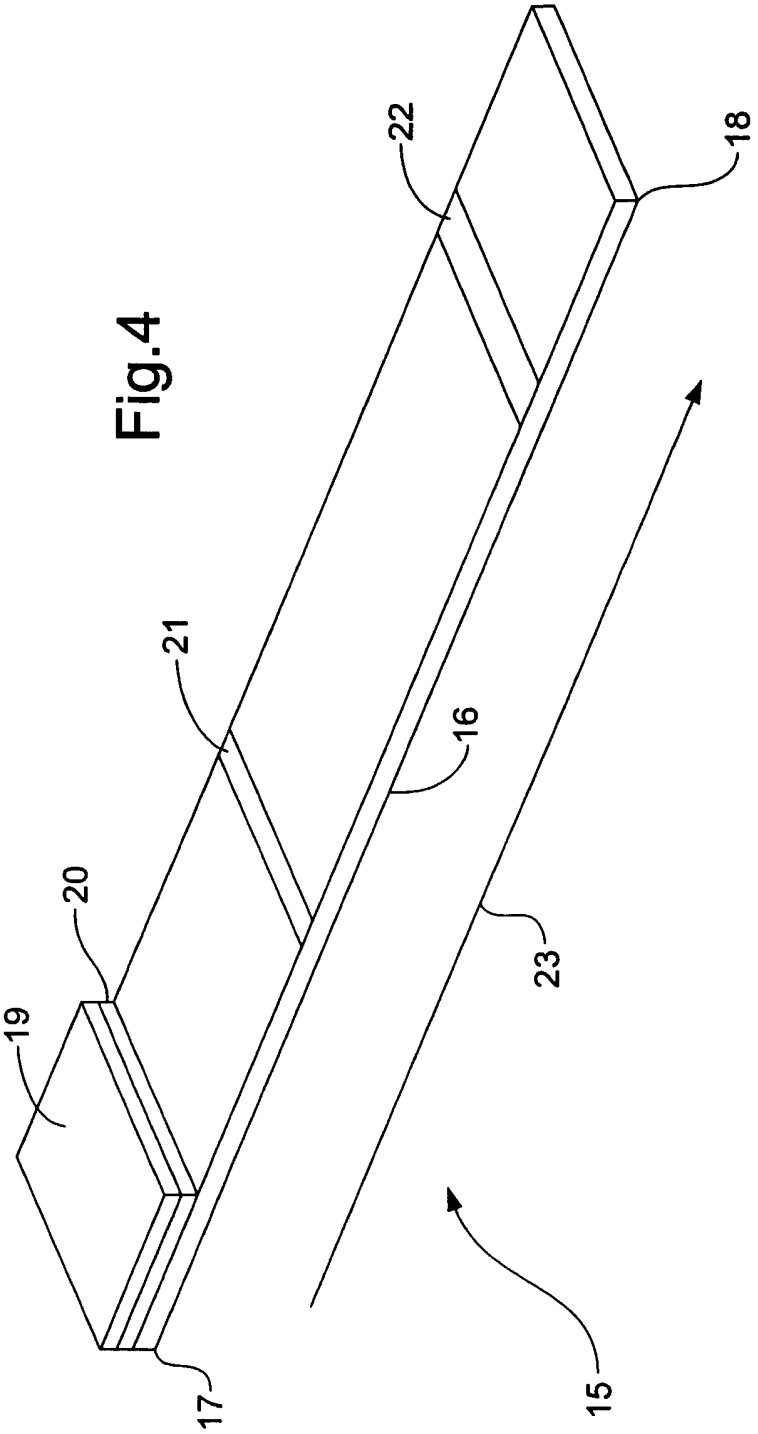


Fig.4



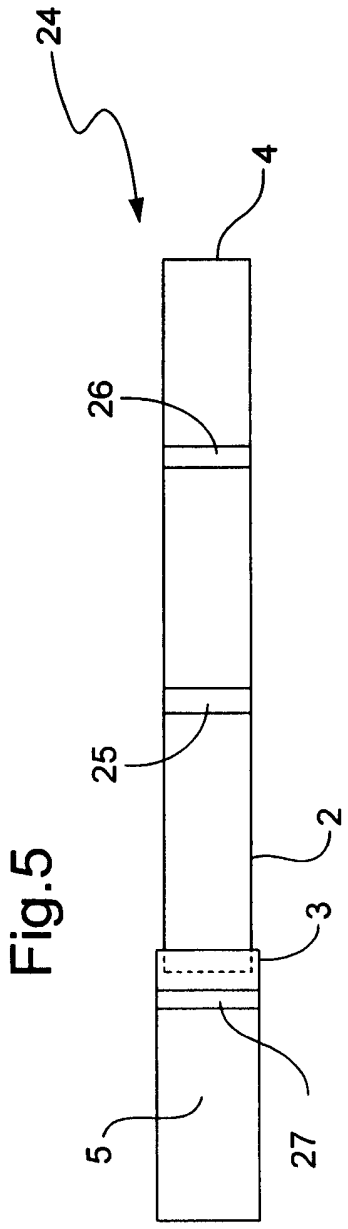


Fig.5

A PROTEASE DETECTION PRODUCT

The present invention relates to a protease detection product which is suitable for detecting a protease enzyme in a sample.

Proteases are enzymes which digest proteins, in particular by hydrolyzing and cleaving peptide bonds. Thus a protein which is degraded by a protease is cleaved into two or more smaller peptides. Cleavage of a peptide occurs when proteases hydrolyze a peptide bond which is adjacent to a specific series of amino acid residues, although other proteases are less specific, and require only one or two amino acids to direct peptide cleavage activity.

An entirely separate area of development has been that of lateral flow immunoassays. These immunoassays allow the detection of analytes in samples. A common example of such an immunoassay is a pregnancy testing kit, which comprises a nitrocellulose strip on which are located, from one end to the other: an absorbent sample receiving area; a labelled antibody band; a capture antibody band and a control band.

The labelled antibody band comprises a plurality of monoclonal antibodies, specific for hCG (human chorionic gonadotropin) and conjugated to gold particles. The labelled antibodies are mobile in, and flow through, the nitrocellulose strip in the presence of a liquid. The capture antibody band comprises a plurality of immobilised monoclonal antibodies, each specific to hCG (although a different epitope thereof from the labelled antibodies). The control band comprises a plurality of immobilised antibodies specific for immunoglobulin G (IgG) from a particular species.

In order to use the kit to test for pregnancy in an individual, a sample (typically urine) from the individual is deposited on the sample receiving zone. The sample naturally flows (following capillary force) along the length of the strip towards firstly the labelled antibody band, then the capture antibody band and finally the control band. It is to be appreciated that the strip thus provides a liquid flow path for the sample.

As the contents of the sample pass the labelled antibody band, the labelled antibody is mobilised within the strip and is also carried in the direction of the capture antibody band. If hCG is present in the sample then the labelled antibody binds to the hCG.

When the labelled antibody reaches the capture antibody band, one of two things may happen. If hCG is present in the sample then the hCG is bound by the capture antibodies. The majority of the hCG will already be bound to the labelled antibodies and thus a complex is formed at the immobilised capture antibody, the hCG forming a link or bridge between the capture antibody and the labelled antibody. This is commonly known as an antibody "sandwich". Alternatively, if there is no hCG in the sample then the labelled antibody passes through the capture antibody band, without interacting with the capture antibodies. It is to be appreciated that the capture antibodies are immobilised and so in either case they do not flow along the strip.

Subsequently, the sample reaches the control band. Because there is an excess of labelled antibody in the kit, even if hCG is present in the sample, there is sufficient labelled antibody present in the kit to ensure some material passes beyond the capture antibody band. Thus, whether or not hCG is present in the sample, some labelled antibody reaches the control band. At the control band, the immobilised anti-IgG antibody binds to, and thus immobilises, the labelled antibody.

It is to be understood that where the labelled antibody is immobilised and concentrated, the presence of the gold particles forms a visible line. Thus, if hCG is present in the sample (which will be the case if the individual is pregnant) then a visible line forms at the capture antibody band. Irrespective of whether or not hCG is present in the sample, a visible line will form at the control band. The line at the control band is useful because it is indicative that the assay has reached its conclusion (which may not occur if, for example, there is insufficient liquid in the sample) and confirms the device has functioned correctly. It also permits a comparison of the capture antibody band with the control band in order to provide additional certainty to the result.

The present invention provides an improved product for detecting the presence of protease enzymes.

According to the present invention, there is provided a protease detection product for detecting a protease enzyme in a sample comprising:

a medium providing a liquid flow path;

a labelled component located on the liquid flow path, the labelled component comprising a base element connected to a releasable element via a protease-sensitive linker peptide, the releasable element comprising a label;

a capture component located downstream of the labelled component on the liquid flow path, the capture component being capable of binding the releasable element; and

immobilisation means for immobilising the intact labelled component.

Conveniently, the immobilisation means comprises an attachment between the base element and a structure on the liquid flow path.

Alternatively, the immobilisation means comprises a further capture component located downstream of the labelled component, the further capture component being capable of binding: the amino acid sequence spanning the protease-sensitive linker peptide; a structural epitope of the linker peptide disrupted by the protease enzyme activity; or a sequence located so that the linker peptide is between the sequence and the label.

Advantageously, the capture component is upstream of the immobilisation means in the liquid flow path.

Alternatively, the capture component is downstream of the immobilisation means in the liquid flow path.

Conveniently, the protease detection product further comprises a protease inhibitor located upstream of the capture component or capture components on the liquid flow path.

Preferably, the protease detection product further comprises a degradable or disruptable barrier on the liquid flow path. For example, in some embodiments, the barrier is a soluble barrier such as collagen or pectin.

Advantageously, the protease detection product further comprises a mixing zone for the mixing of the labelled component and the sample, the soluble barrier being located downstream of the mixing zone and upstream of the capture component or components.

Preferably, the or each capture component comprises one half of a binding pair.

Conveniently, the or each capture component comprises an antibody or an antigen binding fragment thereof.

Alternatively, the or each capture component comprises cellulose, the releasable element further comprising a binding moiety capable of being bound by cellulose.

Preferably, the binding moiety is galactomannan or xyloglucan.

Advantageously, label is a gold particle or a fluorophore group.

In this specification, where reference is made to a component being "on" a liquid flow path, it may equally be "in" the liquid flow path and vice versa.

In order that the present invention may be more fully understood and so that further features thereof may be appreciated, embodiments of the invention will now be described, by way of example, with reference to the accompanying drawings in which:

Figure 1 is a plan view of a protease detection product in accordance with one embodiment of the present invention;

Figure 2 is a schematic view of one component of the protease detection product of Figure 1;

Figure 3 is a schematic view of another component of the protease detection product shown in Figure 1 while in use;

Figure 4 is a perspective view of a protease detection product in accordance with a further embodiment of the present invention; and

Figure 5 is a plan view of a protease detection product in accordance with another embodiment of the present invention.

Referring to Figure 1, a protease detection product 1 comprises a nitrocellulose strip 2 having first and second ends 3, 4. Attached to the first end 3 is a sample receiving zone 5 which comprises an elongate, rectangular absorbent material of slightly greater width than the nitrocellulose strip 2.

Further towards the second end 4 is provided a labelling band 6 which is arranged transverse to the long axis of the strip 2. The labelling band 6 comprises a plurality of labelled components 7 which are shown in further detail in Figure 2. The labelled component 7 comprises a stem 8 which is immobilised within the strip 2 structure to enhance the access of the enzyme to the labelled component 7. Attached to the stem 8 is provided a protease-sensitive linker peptide 9. Attached to the free end of the protease-sensitive linker peptide 9 is provided a label 10, which in this case is a fluorophore but in other embodiments is, for example, a gold particle or a chromogenic moiety.

Further along the strip 2, towards the second end 4, is provided a protease inhibitor band 11, which is located transverse to the long axis of the strip 2. The protease inhibitor band 11 comprises a protease inhibitor dried into the strip 2. It is to be understood that the protease inhibitor band 11 is by no means essential to the invention and is omitted in some other embodiments.

Further along the strip 2 towards the second end 4 is provided a capture band 12, which is also located transverse to the long axis of the strip 2. The capture band 12 comprises a plurality of capture components 13, one of which is shown schematically in Figure 3. In this embodiment, the capture molecule 13 is a monoclonal antibody, immobilised into the strip 2 but this is not essential to the invention. What is important is that each capture component 13 is capable of binding the free end of the peptide linker 9. In Figure 3, the capture component 13 is illustrated binding the peptide linker 9 but it is to be understood that this illustrates the protease detection

product in use. Before the protease detection product 1 is used, no components are bound to the capture component 13.

In order to detect an active protease enzyme in a liquid sample using the protease detection product 1, the sample is deposited on the sample receiving zone 5 and subsequently absorbed into the nitrocellulose strip 2, adjacent the first end 3. A chase buffer comprising a suitable solvent is then deposited on the sample receiving zone in order to ensure that there is sufficient liquid to carry the sample along the strip 2. The sample is then absorbed towards the second end 4 of the strip 2, in the direction of the arrow 14 along what is referred to as the liquid flow path.

When the sample reaches the labelling band 6, any protease enzyme in the sample cleaves the protease-sensitive linker peptide 9 and thus releases the free end of the linker peptide together with the label 10. If, on the other hand there is no active protease in the sample then the linker peptide 9 remains intact and is thus immobilised to the strip 2 via the stem 8.

The sample then continues along the liquid flow path until it reaches the protease inhibitor band 11 at which point any active protease enzyme in the sample is deactivated.

The sample continues along the liquid flow path until it reaches the capture band 12 and the plurality of capture components 13. Since any active protease enzyme in the sample has been deactivated, the sample does not degrade the capture component 13 itself.

If an active protease was in the original sample and has thus cleaved the protease-sensitive linker peptide 9 then the free end of the linker peptide 9 binds to the capture component 13 and remains immobilised on the strip 2. If, on the other hand, there was no active protease in the original sample or any protease in the sample was not capable of cleaving the specific linker peptide 9 then the capture component 13 has nothing to which to bind.

Subsequently, the capture band 12 is visualised. If the label is a fluorophore, as in the present embodiment, then this is carried out under ultraviolet light but if the label

is visible under natural light (for example if the label is a gold particle) then ultraviolet light is unnecessary. If the peptide linker 9 has been cleaved and is thus bound to the capture component 13 then the accumulation of the label 10 at the capture band 12 is visible as a line on the strip 2. If, on the other hand, the sample did not contain a protease to which the peptide linker 9 was sensitive then there is no accumulation of the label 10 at the capture band 12. Thus the presence of an active protease enzyme in the sample can be assessed by observing the presence or absence of a line at the capture band 12, once the assay has completed.

It is to be appreciated that if no protease is present in the sample then the label 10 remains at the labelling band 6. Thus in some alternative embodiments, instead of determining the presence of a line of the label at the capture band 12, the presence of a line at the labelling band 6 is measured. If the label is present at the labelling band 6 then it is indicative of the absence of protease in the sample but if the label is not present at the labelling band 6 or is present at a reduced intensity then it is indicative of the presence of a protease in the sample.

In further embodiments of the present invention, the quantity of the label at the labelling band 6 and the capture band 12 (in other words the intensity of the line of the respective bands 6, 12) are both determined in order assess the ratio of the label at the two bands. By determining the ratio of the label at the two bands, it is possible to quantify the amount of, or activity of, the protease enzyme in the sample. More specifically, the greater the amount of label at the capture band 12 compared with the labelling band 6, the more protease enzyme, or the more active the protease enzyme is in the sample and *vice versa*.

In the above-described embodiment of the invention, the capture component is an antibody. However, in further embodiments of the present invention, the capture components are components other than antibodies. For example, in some embodiments, the protease-sensitive peptide contains a carbohydrate moiety (displaying a CIS diol) and the capture component is phenylboronic acid, which is capable of binding the carbohydrate. In one particularly preferred embodiment, the capture component 13 is cellulose and the protease-sensitive linker peptide 9 is conjugated to galactomannan or xyloglucan, either of which will bind to cellulose.

The advantage of such embodiments is that the protease inhibitor band 11 is not required because cellulose, galactomannan and xyloglucan are not affected by protease enzymes. Other examples of binding pairs that may be used are lectin (e.g. lectin from elderberry) with a carbohydrate (a sugar O-linked to the peptide); and streptavidin with biotin

Referring now to Figure 4, a second embodiment of the present invention is shown. A protease detection product 15 comprises a nitrocellulose strip 16 having first and second ends 17, 18. At the first end of the nitrocellulose strip 16 is located a sample receiving zone 19 which comprises a rectangular sheet of an absorbent material which sits above the strip 16. Impregnated in the sample receiving zone 19 is a protease-sensitive peptide conjugated to a label such as a fluorophore group. The protease sensitive peptide has a cleavage sequence at which the protease can cleave the peptide. Sandwiched between the nitrocellulose strip 16 and the sample receiving zone 19 is a soluble barrier 20 made from, for example, pectin or polyvinyl alcohol. The soluble barrier 20 is temporarily impermeable to liquid and dissolves in the presence of water and thus becomes permeable after a predetermined period of time.

Further along the nitrocellulose strip 16, towards the second end 18 is provided a first capture band 21 which is located transverse to the long axis of the strip 16. The first capture band 21 comprises a plurality of monoclonal antibodies, each immobilised on the surface of the strip 16. The antibodies in the first capture band 21 are specific for a binding sequence of the peptide located such that the cleavage sequence is between the binding sequence and the label. In some alternative embodiments, the antibodies of the first capture band 21 are specific for the cleavage sequence itself or are specific for a conformational epitope that is disrupted through the action of the protease enzyme.

Further along the strip 16 towards the second end 18 thereof is provided a second capture band 22, which is also located transverse to the long axis of the strip 16. The second capture band comprises a plurality of monoclonal antibodies, each immobilised into the surface of the strip 16. The antibodies in the second capture

band 22 are specific for a sequence on the peptide between the cleavage sequence and the label.

In order to use the protease detection product 15 to detect the presence of an active protease enzyme in an aqueous sample, the sample is deposited onto the sample receiving zone 19. A chase buffer, as described in relation to the previous embodiment, is also deposited on the sample receiving zone 19. The sample soaks through the sample receiving zone 19 but is initially incapable of being absorbed further because of the presence of the soluble barrier 20. After a period of time, the buffer in the sample dissolves the soluble barrier 20. During that period of time, however, the sample thoroughly mixes with the protease-sensitive peptide therein and any protease present in the sample will cleave the peptide at the cleavage sequence.

Once the soluble barrier 20 becomes permeable, the sample, mixed with the protease-sensitive peptide, is absorbed along the nitrocellulose strip 16 in the direction of the arrow 23 along a liquid flow path.

The mixture of sample and peptide then passes through the first capture band 21. If there was any active protease enzyme in the sample capable of cleaving the peptide then the peptide will have been cleaved and the antibody will bind a peptide fragment that does not carry the label. In the alternative embodiments, no peptide will be able to bind to the antibody in the first capture band because the antibody is specific for the sequence across the cleaving point, or is specific for a conformational epitope that is disrupted through the action of the protease enzyme activity. If, on the other hand, there was no active protease in the sample capable of cleaving the peptide then intact peptide will bind to the antibodies in the first capture band 21.

The mixture of peptide and sample then continues along the strip 16 in the direction of the arrow 23 where it reaches the second capture band 22. Whether or not protease was present in the sample, the intact or cleaved peptide will bind to the antibodies in the second capture band 22. It is to be appreciated that if the protease is in the sample then little or no label will become bound to the first capture band 21 so more label is available to be bound to the second capture band 22

The strip 16 is then visualised in order to locate the label on it. If the label is a fluorophore group as in this embodiment, then the strip 16 is visualised under ultraviolet light. Where the label has accumulated, it is visible as a line on the strip 16. If the label has accumulated at the first capture band 21 then this is indicative of the absence of an active protease enzyme in the sample.

In an alternative embodiment, the presence of the label at the second capture band 22 is determined, the presence of label at the second capture band 22 being indicative of the presence of an active protease enzyme in the sample.

In some further embodiments, the presence of label is determined at both the first and second capture bands 21, 22 and the ratio of the intensity of the lines of label at the two bands 21, 22 is assessed. The more label there is at the second capture band 22 as compared with the first capture band 21 being indicative of the more protease enzyme, or a more active enzyme, in the sample and *vice versa*.

It is also to be understood that the second capture band 22 acts as a "control band" because label accumulation at the second capture band 22 indicates that the sample has penetrated the soluble barrier 20 and has reached the end of the assay.

In some alternative embodiments, the location of the first and second capture bands, 21, 22 is reversed, i.e. the second capture band 22 is upstream of the first capture band.

It is to be appreciated that in the embodiment shown in Figure 5, the advantage of providing the soluble barrier 20 is that thorough mixing of the protease-sensitive peptide and the sample can occur before the mixture proceeds along the liquid flow path. If the soluble barrier 20 were not provided then the nitrocellulose strip 16 would have to be much longer in order to provide sufficient time for the mixing to occur and the protease to cleave the peptide before the mixture reached the first capture band 21.

Referring, now, to Figure 5, a variant of the embodiment shown in Figure 1 is depicted with like components give the same reference numerals. In this embodiment, a protease detection product 24 differs from the product shown in

Figure 1 in that the labelling band 6 and the capture band 12 are replaced with a capture band 25 and a further capture band 26, respectively. Furthermore, the protease inhibitor band 11 is omitted and a soluble barrier 27 is provided in the sample receiving zone 5, separating the main part of the sample receiving zone 5 from the first end 3 of the nitrocellulose strip 2.

Dried into the main part of the sample receiving zone 5 is a protease-sensitive peptide conjugated to a label such as a fluorophore group. The protease-sensitive peptide has a cleavage sequence at which a protease can cleave the peptide.

The capture band 25 comprises a plurality of monoclonal antibodies, each immobilised onto the surface of the strip 2. The antibodies in the capture band 25 are specific for a sequence on the peptide between the cleavage sequence and the label.

The further capture band 26 comprises a plurality of monoclonal antibodies, each immobilised on the surface of the strip 2. The antibodies in the further capture band 26 are specific for a binding sequence of the peptide located such that the cleavage sequence is between the binding sequence and the label. In some alternative embodiments, the antibodies of the further capture band 26 are specific for the cleavage sequence itself or are specific for a conformational epitope which is disrupted through the action of the protease enzyme.

The protease detection product 24, is used as in the previous embodiments. Thus a sample is deposited on the sample receiving zone 5 and a chase buffer is added. The sample then mixes with the protease sensitive peptide on the sample receiving zone 25 while the soluble barrier 27 is dissolved by the buffer. If there is a suitable protease in the sample then the protease-sensitive peptide is cleaved.

After a period of time, the soluble barrier 27 is degraded and the mixture of sample, buffer and protease-sensitive peptide passes along the strip 2 from the first end 3 to the second end 4. As the mixture passes the capture band 25, the antibodies in the capture band 25 bind to the protease-sensitive peptides, irrespective of whether or not it has been cleaved by a protease. However, because there is an excess of the protease-sensitive peptide provided in the sample receiving zone 5, some of the

protease-sensitive peptide passes beyond the capture band 25 and reaches the further capture band 26.

At the further capture band 26, the antibodies bind and immobilise the binding sequence of the peptide. If the protease-sensitive peptide has been cleaved then the label is no longer attached to the binding sequence and so the label is not immobilised at the further capture band 26. In the alternative embodiments, the peptide does not bind to the antibodies of the further capture band 26, at all, if there has been cleavage of the protease-sensitive peptide and so for this reason the label does not accumulate at the further capture band 26. If, on the other hand, the protease-sensitive peptide has not been cleaved then the label does accumulate at the further capture band 26.

Once the sample has passed the further capture band 26, the strip 2 is inspected to determine the outcome of the assay. The level of label which accumulates at the further capture band 26 is compared with the level of label accumulated at the capture band 25. The lower the level of label at the further capture band 26, the greater the amount, or the greater the activity of the protease in the sample (and *vice versa*) since it is indicative of a greater amount of cleavage of the protease-sensitive peptide.

It is to be appreciated that in the other versions of this embodiment, the position of the capture band and the further capture band 26 may be reversed with respect to each other (i.e. the further capture band 26 may be located upstream on the liquid flow path of the capture band 25).

In one particular version of this embodiment, the label comprises a gold particle to which is conjugated the protease-sensitive peptide. Also conjugated to the gold particle is a galactomannan moiety. The capture band 25 comprises a plurality of antibodies capable of binding a binding sequence on the peptide, the cleavage sequence being between the gold particle and the binding sequence. The further capture band 26 comprises PBA, which binds carbohydrate displaying a CIS diol. In the absence of protease, gold particles are bound at and accumulate at both the capture band 25 and the further capture band 26. In the presence of a suitable

protease, however, the cleavage sequence is cleaved and so any peptide bound at the first capture band 25 is not attached to a gold particle. Thus gold particles only accumulate at the further capture band 26 in the presence of the protease. Accordingly, the presence or absence of an active protease enzyme in the sample can be assessed by comparing the accumulation of the gold particle at the capture band 25 with gold particle accumulation at the further capture band 26.

CLAIMS

1. A protease detection product for detecting a protease enzyme in a sample comprising:

a medium providing a liquid flow path;

a labelled component located on the liquid flow path, the labelled component comprising a base element connected to a releasable element via a protease-sensitive linker peptide, the releasable element comprising a label;

a capture component located downstream of the labelled component on the liquid flow path, the capture component being capable of binding the releasable element; and

immobilisation means for immobilising the intact labelled component.

2. A protease detection product according to claim 1 wherein the immobilisation means comprises an attachment between the base element and a structure on the liquid flow path.

3. A protease detection product according to claim 1 wherein the immobilisation means comprises a further capture component located downstream of the labelled component, the further capture component being capable of binding: the amino acid sequence spanning the protease-sensitive linker peptide; a structural epitope of the linker peptide disrupted by the protease enzyme activity; or a sequence located so that the linker peptide is between the sequence and the label.

4. A protease detection product according to any one of the preceding claims wherein the capture component is upstream of the immobilisation means in the liquid flow path.

5. A protease detection product according to any one of the preceding claims wherein the capture component is downstream of the immobilisation means in the liquid flow path.

6. A protease detection product according to any one of the preceding claims further comprising a protease inhibitor located upstream of the capture component or capture components on the liquid flow path.

7. A protease detection product according to any one of the preceding claims further comprising a degradable or disruptable barrier on the liquid flow path

8. A protease detection product according to claim 7 further comprising a mixing zone for the mixing of the labelled component and the sample, the soluble barrier being located downstream of the mixing zone and upstream of the capture component or components.

9. A protease detection product according to any one of the preceding claims wherein the or each capture component comprises one half of a binding pair.

10. A protease detection product according to claim 9 wherein the or each capture component comprises an antibody or an antigen binding fragment thereof.

11. A protease detection product according to claim 9 wherein the or each capture component comprises cellulose, the releasable element further comprising a binding moiety capable of being bound by cellulose.

12. A protease detection product according to claim 11 wherein the binding moiety is galactomannan or xyloglucan.

13. A protease detection product according to any one of the preceding claims wherein the label is a gold particle or a fluorophore group.

14. A protease detection product substantially as herein described with reference to and as shown in the accompanying figures.

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Application No: GB0607070.0 **Examiner:** Dr Jonathan Corden

Claims searched: 1-13 **Date of search:** 10 August 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
A	-	US 5741659 A (RALLS et al) see abstract and whole document
A	-	GB 2259362 A (BIOSYN) see abstract and whole document
A	-	US 5932410 A (WHITTAKER et al) see abstract and whole document
A	-	WO 2004/048935 A2 (MARLIGEN) see abstract and whole document
A	-	WO 2005/012558 A1 (UNIV. OF EDINBURGH) see abstract and whole document
A	-	WO 2005/005657 A1 (MOLECULAR SENSING) see abstract and whole document
A	-	US 2004/0096926 A1 (PACKARD et al) see abstract and whole document
A	-	US 2003/211560 A1 (PROMEGA) see abstract and whole document

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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