

SUMMARY

Disclosed is a method of generating an antibody to a protein, said method comprising:

- 5 (i) identifying an antigenic epitope in said protein by exposing the protein to limited or restricted proteolysis by contacting the protein with at least one protease to form at least one digested, deconstructed or truncated version of the protein and at least one surface-exposed peptide that is cleaved off from the protein by the action of said protease and generating an antigenic epitope based on said surface-exposed peptide; and
- 10 (ii) raising an antibody against the antigenic epitope.

Disclosed is a method of generating an antibody to a protein, said method comprising:

- 15 (i) exposing the protein to limited or restricted proteolysis by contacting the protein with at least one protease to form at least one digested, deconstructed or truncated version of the protein and at least one surface-exposed peptide that is cleaved off from the protein by the action of said protease; and
- (ii) identifying an antigenic epitope by identifying a surface-exposed epitope among the at least one surface-exposed peptide that is present in a region of the protein that results in a lack of, or significantly altered, biological function of the protein when the peptide is cleaved off or removed from the protein during the limited or restricted proteolysis; or
- 20 selecting at least one target region within the protein based on bioinformatics and/or known data of biological function of the protein and identifying an antigenic epitope by identifying a surface-exposed epitope among the at least one surface-exposed peptide present in said at least one target region; and
- 25 (iii) raising an antibody against the antigenic epitope.

Disclosed is a method of identifying an antigenic epitope, said method comprising:

- 30 (i) exposing a protein to limited or restricted proteolysis by contacting the protein with at least one protease to form at least one digested, deconstructed or truncated version of the protein and at least one surface-exposed peptide that is cleaved off from the protein by the action of said protease; and

- (ii) identifying an antigenic epitope by identifying a surface-exposed epitope among the at least one surface-exposed peptide that is present in a region of the protein that results in a lack of, or significantly altered, biological function of the protein when the peptide is cleaved off or removed from the protein during the limited or restricted proteolysis; or
 selecting at least one target region within the protein based on bioinformatics and/or known data of biological function of the protein and identifying an antigenic epitope by identifying a surface-exposed epitope among the at least one surface-exposed peptide present in said at least one target region.

In one aspect, there is provided a method of identifying an epitope on a protein that can be bound by an antibody, said method comprising:

- (i) identifying sites at which one or more protease has cut said protein after said protein has been exposed to limited or restricted proteolysis by said one or more protease; and
- (ii) probing a plurality of epitopes on said protein that are between the cut sites, that overlap with a cut site, or that are in a region that flanks a cut site with antibodies directed to said epitopes, thereby identifying one or more epitopes that can be bound by an antibody.

In another aspect, there is provided a method of identifying an epitope on a protein that can be bound by an antibody, said method comprising:

- (i) performing in silico protease digestion of said protein with one or more proteases to identify sites on the protein that are predicted to be cut by said one or more proteases;
- (ii) performing in vitro limited or restricted proteolysis of said protein with one or more proteases;
- (iii) identifying peptides released from said protein by the in vitro protease digestion of step (ii), thereby identifying cut sites;
- (iv) comparing the in silico predicted cut sites identified in step (i) with cut sites identified in step (iii);
- (v) probing one or more epitopes in a region of the protein containing or flanking a cut site that is an in silico predicted protease cut site identified in step (i) but that is not a cut site identified in step (iii) with one or more antibodies; and
- (vi) identifying whether or not said one or more antibodies bind to said one or more epitopes thereby identifying an epitope on a protein that can be bound by an antibody.

The present disclosure relates to methods of detecting and identifying amino acid sequences in proteins where said amino acid sequences are well-exposed, and functionally relevant, at least they are well-exposed. These amino acid sequences which we refer to as "hot spots", thus, may be utilized as antigenic epitopes that guides antibody targeting, discovery, and development. Furthermore, these amino acid sequences can be ranked based on their appearance after proteolytic digestion, and based on functional relevance from already known bioinformatic data or from functional/pharmacological testing. Thus, from a list of several amino acid sequences resulting from a proteolytic digestion, the best suited amino acid sequences (based on functional and structural arguments) can be picked for antigenic epitope discovery and development. The proteolytic digestion is performed under limiting conditions, i.e. the activity of the protease or several proteases is very low such that just one or a few surface-exposed peptides are cleaved off from the target protein at a time. The proteases are thus used as druggability probes for antibody binding to a target protein.

In an embodiment, the antibodies are pharmacologically active. In another embodiment, the antibodies are pharmacologically active and developed for therapeutic usage. More specifically, such methods include proteomic tools to reveal hot spot epitopes of target proteins.

In an embodiment of the disclosure, a protein is digested, deconstructed and/or truncated through protease action and all well-exposed amino acid sequences are used for antigenic epitope generation, and antibodies developed based on said antigenic epitopes are tried for potency, efficacy, pharmacological profiling, and other testing as customary in antibody discovery used in the pharmaceutical industry.

In an embodiment of the disclosure, a protein is digested, deconstructed and/or truncated through protease action and in parallel probed by a functional assay on the digested, deconstructed

and/or truncated protein to delineate functionally important regions of the protein. The relevant protein is sometimes denoted target protein herein.

- 5 In an embodiment, the digestion, deconstruction and/or truncation of the target protein is performed in parallel by a functional assay to delineate functionally important regions of the target protein to guide epitope selection for antibody generation.

In an embodiment, the digestion, deconstruction and/or truncation, and functional assay of
10 digested, deconstructed and/or truncated protein and native target protein are combined with other bioinformatic and otherwise known facts about protein function to delineate functionally important regions of the target protein to guide epitope selection for antibody generation.

In an embodiment, a single protease may be used to digest, deconstruct and/or truncate the
15 target protein. In another embodiment, multiple proteases may be used to digest, deconstruct and/or truncate the target protein, sequentially one at a time or in parallel. Such proteases are exemplified but not limited to Arg-C proteinase, Asp-N endopeptidase, Clostripain, Glutamyl endopeptidase, Lys-C, Lys-N, Trypsin, Chymotrypsin, Proteinase K and Thermolysin. A region that is easily digested by several proteases should be located in
20 an exposed region of the protein and a region that is only digested by a single protease is probably located in a more hidden region. Alternatively, the protease has unique cleaving specificity or/and physicochemical properties or/and structural features such that it can identify surface-exposed peptides on a target protein that other proteases cannot. Thus, the usage of multiple proteases is preferable, and each different protease can yield
25 complementary or unique information about surface-exposed peptides suitability as antigenic epitopes.

The embodiments enable new methodology/technology for rapid and precise development of pharmacologically active antibodies that can be used for pharmacological studies, e.g.
30 they can be used as a tool for detecting biological compounds in e.g. cell or *in vitro* assays. More importantly, said antibodies may be used to treat a medical condition in humans and animals. The embodiments can be applied to all proteins, soluble or membrane bound, extracellular or intracellular. The embodiments can furthermore be exploited to yield new fundamental understanding of protein function.

- 35 The present disclosure also provides an antibody generated by a method of the present disclosure.

In another aspect, there is provided an antibody that binds to an epitope as described herein.

The present disclosure also provides an antigenic epitope identified by a method of the present disclosure.

In another aspect, there is provided an epitope identified by the method as described herein.

The present disclosure also provides an antibody against an antigenic epitope of the present
5 disclosure.

Other features and advantages of the disclosure will be apparent from the following detailed description.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The embodiments, together with further objects and advantages thereof, may best be understood by making reference to the following description taken together with the accompanying drawings, in which:

15 Figure 1

Peptides detected from TRPV1 after limited proteolysis with 5 μ g/ml trypsin at room temperature, n=6. A: Location of detected peptides shown in a 3D-model of TRPV1.

Peptides were detected after 0.5 min (magenta), 5 min (orange), and 15 min (blue) B:

Location of detected peptides shown in a schematic representation of TRPV1. Peptides

20 were detected after 0.5 min (magenta), 5 min (orange), and 15 min (blue). C: Bar plot of detected peptides digested from TRPV1 after limited proteolysis with 5 μ g/ml trypsin, showing at which time points they were confirmed.

Figure 2

25 Peptides digested from TRPV1 after 5 min exposure to 5 μ g/ml, 20 μ g/ml or 40 μ g/ml trypsin (Tr) and the change in current response after their removal. A-C: Location of digested peptides from TRPV1, showing peptides digested within the flow cell (cyan) and peptides digested within the flow cell followed by a complete digestion overnight (yellow). D: Representative traces of inside-out recordings of TRPV1 when activated with 1 μ M

30 capsaicin (Cap), followed by 5 min exposure to either buffer or trypsin and an additional activation with capsaicin. From top to bottom: 5 min exposure to buffer, 5 μ g/ml, 20 μ g/ml and 40 μ g/ml trypsin respectively. Traces have been digitally filtered at 100 Hz for figure presentation purposes only.

35 Figure 3

Electrophysiological patch clamp recordings of TRPV1 function showing the current trace time integral for the second activation with capsaicin, calculated as a percentage of the

CLAIMS

1. A method of identifying an epitope on a protein that can be bound by an antibody, said method comprising:
 - 5 (i) identifying sites at which one or more protease has cut said protein after said protein has been exposed to limited or restricted proteolysis by said one or more protease; and
 - (ii) probing a plurality of epitopes on said protein that are between the cut sites, that overlap with a cut site, or that are in a region that flanks a cut site with
 - 10 antibodies directed to said epitopes, thereby identifying one or more epitopes that can be bound by an antibody.
2. The method of claim 1, wherein a single protease is used.
- 15 3. The method of claim 1, wherein multiple proteases are used.
4. The method of any one of claims 1 to 3, wherein said protease is selected from the group consisting of: trypsin, Arg-C proteinase, Asp-N endopeptidase, Clostripain, Glutamyl endopeptidase, Lys-C, Lys-N, Chymotrypsin, Proteinase K, Thermolysin,
 - 20 Pepsin, Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10, Enterokinase, Factor Xa, GranzymeB, Neutrophil elastase, Proline-endopeptidase, Staphylococcal peptidase I, and Thrombin.
- 25 5. The method of any one of claims 1 to 4, wherein said protease is selected from the group consisting of: trypsin, Asp-N endopeptidase, Chymotrypsin, pepsin and Proteinase K.
6. The method of any one of claims 1 to 5, wherein said protein is a membrane protein
 - 30 that is present in a proteoliposome derived from cells.
7. The method of claim 6, wherein said proteoliposome is immobilized in a flow cell to create a stationary phase of membrane proteins.
- 35 8. The method of any one of claims 1 to 7, wherein said sites at which one or more protease has cut said protein are identified using mass spectrometry.

9. The method of any one of claims 1 to 8, wherein said method further comprises a step prior to step (ii) of generating a plurality of isolated epitopes having sequences that correspond to epitopes on said protein that are between the cut sites, that overlap with a cut site, or that are in a region that flanks a cut site, and generating
5 antibodies that bind to said isolated epitopes, and using said antibodies in step (ii) for probing said plurality of epitopes on said protein.
10. The method of any one of claims 1 to 9, wherein said epitope is within 20 amino acids of said cut site.
- 10 11. The method of any one of claims 1 to 10, wherein said plurality of epitopes is a set of epitopes wherein the sequence of each epitope in the set is offset from another epitope in the set by 1, 2 or 3 amino acids.
- 15 12. The method of any one of claims 1 to 11, wherein said method further comprises a step of producing an antibody against an epitope identified by any one of claims 1 to 11.
13. A method of identifying an epitope on a protein that can be bound by an antibody,
20 said method comprising:
 - (i) performing *in silico* protease digestion of said protein with one or more proteases to identify sites on the protein that are predicted to be cut by said one or more proteases;
 - 25 (ii) performing *in vitro* limited or restricted proteolysis of said protein with one or more proteases;
 - (iii) identifying peptides released from said protein by the *in vitro* protease digestion of step (ii), thereby identifying cut sites;
 - (iv) comparing the *in silico* predicted cut sites identified in step (i) with cut sites identified
30 in step (iii);
 - (v) probing one or more epitopes in a region of the protein containing or flanking a cut site that is an *in silico* predicted protease cut site identified in step (i) but that is not a cut site identified in step (iii) with one or more antibodies; and
 - (vi) identifying whether or not said one or more antibodies bind to said one or more
35 epitopes thereby identifying an epitope on a protein that can be bound by an antibody.

14. The method of claim 13, further comprising performing protein homology modelling to predict which *in silico* predicted protease cut sites are likely to be exposed.
15. The method of claim 13 or claim 14, further comprising performing *in silico* docking of antibody fragments or proteases to predict which *in silico* predicted cut sites are likely to be cut *in vitro*.
16. The method of any one of claims 13 to 15, wherein a single protease is used.
- 10 17. The method of any one of claims 13 to 15, wherein multiple proteases are used.
18. The method of any one of claims 13 to 17, wherein said protease is selected from the group consisting of: trypsin, Arg-C proteinase, Asp-N endopeptidase, Clostripain, Glutamyl endopeptidase, Lys-C, Lys-N, Chymotrypsin, Proteinase K, Thermolysin, Pepsin, Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10, Enterokinase, Factor Xa, GranzymeB, Neutrophil elastase, Proline-endopeptidase, Staphylococcal peptidase I, and Thrombin.
- 20 19. The method of any one of claims 13 to 18, wherein said protease is selected from the group consisting of: trypsin, Asp-N endopeptidase, Chymotrypsin, pepsin and Proteinase K.
20. The method of any one of claims 13 to 19, wherein said protein is a membrane protein that is present in a proteoliposome derived from cells.
21. The method of claim 20, wherein said proteoliposome is immobilized in a flow cell to create a stationary phase of membrane proteins.
- 30 22. The method of any one of claims 13 to 21, wherein identifying peptides released from said protein by the *in vitro* protease digestion of step (ii), thereby identifying cut sites, is done by mass spectrometry.
23. The method of any one of claims 13 to 22, wherein said method further comprises a step prior to step (v) of generating one or more isolated epitopes having sequences that correspond to one or more epitopes on said protein that are in a region of the protein containing or flanking a cut site that is an *in silico* predicted protease cut site

identified in step (i) but that is not a cut site identified in step (iii), and generating antibodies that bind to said isolated epitopes, and using said antibodies in step (v) for probing said one or more epitopes of said protein.

- 5 24. The method of any one of claims 13 to 23, wherein a plurality of epitopes is probed.
25. The method of any one of claims 13 to 24, wherein said epitope is within 20 amino acids of said cut site that is an *in silico* predicted protease cut site identified in step (i) but that is not a cut site identified in step (iii).
- 10 26. The method of any one of claims 13 to 25, wherein a plurality of epitopes is probed and said plurality of epitopes is a set of epitopes wherein the sequence of each epitope in the set is offset from another epitope in the set by 1, 2 or 3 amino acids.
- 15 27. The method of any one of claims 13 to 26, wherein said method further comprises a step of producing an antibody against an epitope identified by any one of claims 13 to 26.
28. An epitope identified by the method of any one of claims 1 to 11 and 13-26.
- 20 29. An antibody that binds to an epitope of claim 28.