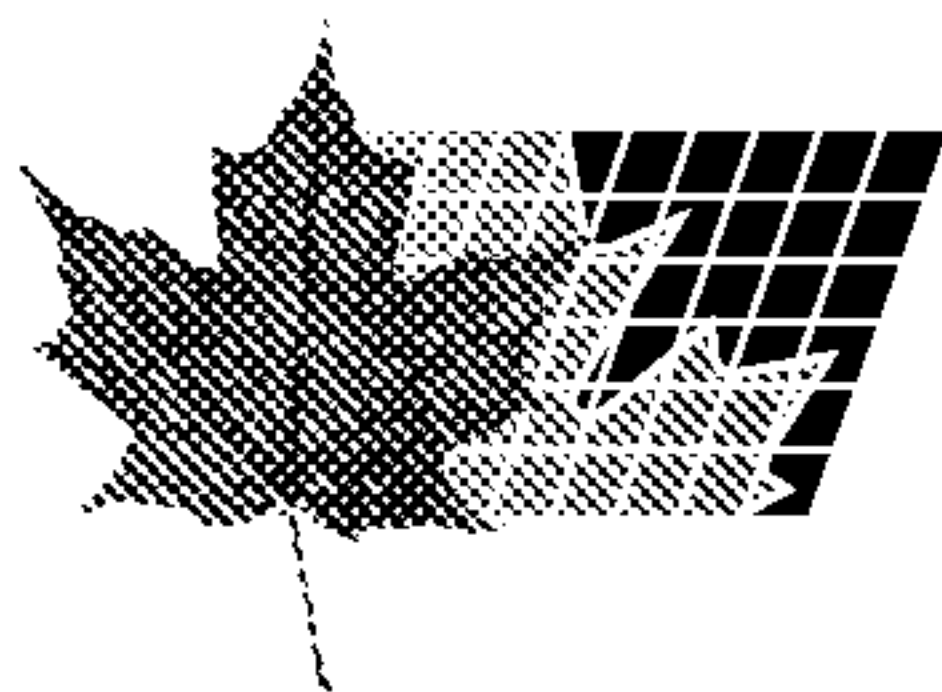




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(54) **DISPOSITIF ET METHODE POUR LA DETERMINATION DES
LIMITES DU DOMAINE DES PROTEINES**

(54) **A DEVICE AND METHOD FOR THE DETERMINATION OF
PROTEIN DOMAIN BOUNDARIES**

(57) A method and device to assist in the determination of protein domain boundaries is described. In particular the device is designed to provide a high throughput of proteolytic digestion of proteins to identify domains and their boundaries, for use in protein structure determination, in a manner that is amenable to automation. Proteases are immobilized in a convenient format such as a microtitre plate and preferably arranged in a matrix thereby allowing for simultaneous degradation of a protein by a number of proteases at a number of concentrations.

ABSTRACT

A method and device to assist in the determination of protein domain boundaries is described. In particular the device is designed to provide a
5 high throughput of proteolytic digestion of proteins to identify domains and their boundaries, for use in protein structure determination, in a manner that is amenable to automation. Proteases are immobilized in a convenient format such as a microtitre plate and preferably arranged in a matrix thereby allowing for simultaneous degradation of a protein by a
10 number of proteases at a number of concentrations.

A DEVICE AND METHOD FOR THE DETERMINATION OF PROTEIN DOMAIN BOUNDARIES

FIELD OF INVENTION

5 The present invention relates to methods for the structural determination of proteins.

BACKGROUND OF INVENTION

10 The molecular structure of proteins allows them play crucial roles in virtually all biological processes, including: enzymatic catalysis, transport and storage; coordinated motion; mechanical support; immune protection; generation and transmission of nerve impulses; and control of growth and differentiation. In particular, the side chains of the different amino acids that
15 comprise proteins, enables these long macromolecules to fold into distinctive structures and form complementary surfaces and clefts and enables them to specifically recognize and interact with highly diverse molecules. The catalytic power of enzymes comes from their capacity to bind substrates in precise orientations and to stabilize transition states in the making and breaking of chemical bonds. Conformational changes transmitted between distant sites in protein molecules
are at the heart of the capacity of proteins to transduce energy and information. Thus, the three dimensional structure of a protein is the key to its ability to function in virtually all biological processes.

20 Discussions pertaining to protein architecture refer to four levels of structure. Primary structure is the amino acid sequence. Secondary structure refers to the spatial arrangement of amino acid residues that are near one another in the linear sequence. Some of these steric relationships are of a regular kind, giving rise to a periodic structure. Tertiary structure refers to the spatial arrangement of amino acid residues that are far apart in the linear sequence, and to the pattern of disulfide bonds. The term, quaternary structure, refers to proteins containing more than one

polypeptide chain wherein each polypeptide chain is referred to as a subunit, and the quaternary structure refers to the spatial arrangement subunits and the nature of their contacts.

5 Some polypeptide chains fold into two or more compact regions that may be joined by a flexible segment of polypeptide chain. These compact globular units, called domains, range in size from about 50 to 400 amino acid residues, and they seem to be the modular units from which proteins are constructed. While small proteins may contain only a single domain, larger proteins contain a number of domains, which are often connected by relatively open lengths of polypeptide chain. Although all the information required for the folding of a protein chain is contained in its amino acid sequence, it is not yet known how to "read" this information so as to predict the detailed
10 three-dimensional structure of a protein whose sequence is known. Consequently, the folded conformation can only be determined by an elaborate X-ray diffraction analysis performed on crystals of the protein or, if the protein is very small, by nuclear magnetic resonance techniques.

Determination of the three dimensional structure of proteins is an important area of pure and applied research. The technique of X-ray crystallography utilizes the diffraction of X-rays from
15 crystals in order to determine the precise arrangement of atoms within the crystal. The limiting step in all of these areas of research involves the growth of a suitable crystalline sample. Although considerable advances are being made in the area of high field nuclear magnetic resonance, at the present time, the only method capable of producing a highly accurate three dimensional structure of most proteins is by the application of X-ray crystallography. This
20 requires the growth of reasonably ordered protein crystals (crystals which diffract X-rays to at least 3.0 angstroms resolution or less).

Because of the complexity of proteins, obtaining suitable crystals can be quite difficult. Typically, several hundred to several thousand individual experiments must be performed to determine crystallization conditions, each examining a matrix of pH, buffer type, precipitant
25 type, protein concentration, temperature, etc. This process is extremely time consuming and labor intensive. In this regard, the field is difficult to exploit. The resulting three dimensional structure

produced from the protein crystals can have enormous implications in the fundamental understanding of molecular biology such as how enzymes perform various catalytic activities, switch on biological pathways, or transport molecules within the circulatory system. In the past few years the determination of protein structures important as therapeutic targets has made possible the rational design of new, more effective pharmaceuticals.

Recent advances in this field such as high speed computer graphics and X-ray area detection technologies has revolutionized the pace at which the three-dimensional structures can be determined. Still, however, the bottle neck has been the determination of conditions necessary to grow high quality protein crystals. In order for protein crystals to be suitable for structural analysis via X-ray diffraction methods, crystals on the order of about 0.2 mm in diameter or greater must be obtained depending on the intrinsic quality of the protein crystal, the size of the unit cell, and the flux of the X-ray source, etc. This has proved extremely inconvenient and difficult to accomplish on a consistent basis using techniques at present. In short, the currently accepted practice of screening for protein crystallization conditions suffers from a myriad of problems which have limited the use of high resolution X-ray crystallographic methods in the determination of the three dimensional structures of the protein molecules.

A different strategic approach has been developed to identify protein domains that are amenable to NMR analysis or X-ray crystallography. Different domains may be linked together by intervening sections of polypeptide chain to form the protein molecule. Analysis of a single domain is more easily conducted in isolation from its parent protein. The determination of an individual domain structure facilitates elucidation of the parent structure. Limited proteolysis has been used to isolate and identify stable domains of proteins, which have a high likelihood of being good targets for protein structure determination.

The approach is based upon the observation that low concentrations of one or more specifically chosen proteases cleave proteins into proteolytically stable domains amenable to NMR analysis or crystallography (Morin, P.E; *et al.* Proc. Natl. Acad. Sci. **1996**, 93, 10604 - 10608; Barswell,

J.A; *et al.* J. Bio. Chem. **1995**, 270, 20556 - 20559; Pfuetzner, R.A; *et al.* J. Bio. Chem. **1997**, 272, 430 - 434; and Malhotra, A; *et al.* Cell **1996**, 87, 127 - 136).

Limited proteolysis has been conducted to isolate and identify stable domains of proteins. In this process, the protein is commonly incubated with four to six different proteases at different concentrations for different amounts of time. Typical protease digestion reactions are conducted by dissolving an enzyme in water thereby to allow the enzyme to act on a substrate in an aqueous solution. However, the fact that the enzyme reaction is a homogeneous reaction in an aqueous solution is a great hindrance to performance of continuous reaction in industrial applications and also makes it very difficult to recover remaining active enzymes for repeated use after the reaction. In addition, complicated operational procedures are necessary for separation and purification of the reaction product.

The digestion products can be analyzed by SDS/polyacrylamide gel electrophoresis and proteolytically stable fragments can be identified by approximate mass. These products can then be isolated by reverse phase chromatography and their accurate mass determined by mass spectrometry. The accurate mass of a proteolytic fragment is sufficient to uniquely identify the boundaries of the fragment within a sequence of a protein. The identification of the proteolytic fragment sequence facilitates the recombinant preparation of the domain in sufficient quantities for X-ray and NMR analysis.

A limitation of the aforementioned strategy is the time consuming nature of cleaving, identifying and isolating the domains of a protein from the digestion solution.

SUMMARY OF INVENTION

It is an object of the present invention to provide a device and method for the determination of protein domain boundaries. In accordance with an aspect of the invention there is provided an apparatus comprising a solid support having proteases immobilized in individual compartments

wherein the organization of proteases is organized in a two-dimensional matrix format with: (i) distinct concentrations of each protease are aligned, in increasing concentration, along a first axis; and (ii) different proteases are aligned along a second axis that is perpendicular to the first axis.

5 In accordance with a further aspect of the invention there is provided an automated process for the purification of a proteolytically digested fragment of a protein which comprises the steps: (i) contacting an aliquot of a protein solution or a solution of a protein fragments, with an apparatus comprising a solid support having proteases immobilized in individual compartments wherein the organization of proteases is organized in a two-dimensional matrix format with: (a)
10 distinct concentrations of each protease are aligned, in increasing concentration, along a first axis; and (b) different proteases are aligned along a second axis that is perpendicular to the first axis to yield proteolytically treated fragments; and (ii) subjecting said proteolytically treated fragments to a purification step to yield one or more samples.

15 In view of the foregoing disadvantages in existing methods of determining protein domains in the art, the present invention provides a device and method for conducting limited proteolysis of proteins to determine protein domains useful for structure determination.

In support of the principal object, a further object of the present invention is to provide a high throughput device and method for enzymatically cleaving proteins into domains which allows for a highly efficient means to identify protein domain boundaries for use in protein crystallization,
20 in a manner that is amenable to automation.

In accordance with another aspect of the present invention there is provided an apparatus comprising a solid support, such as a microtiter plate having one or more proteases immobilized in separate compartments. Each individual protease is immobilized at increasing concentrations across the compartments. Optionally, the device can be connected upstream to an automatic

means of adding a protein solution, or a solution of a protein fragment, and downstream to an automatic means of removing and subjecting the proteolytically digested product to a purification step.

In accordance with an aspect of the present invention there is provided a method for determining the boundaries of a proteolytically digested fragment of a protein which comprises the steps:

(i) incubating a protein, or a fragment thereof, with at least one immobilized protease to yield protein fragments; and (ii) subjecting the resulting solution of protein fragments to one or more purification steps to isolate the fragment(s) of interest; (iii) subjecting the isolated fragment(s) to either nanospray or matrix-assisted time-of-flight mass spectrometry; (iv) matching the mass of the proteolytic fragment to the protein sequence.

The present invention also includes a kit containing the device of the present invention together with appropriate reagents and instructions for its use.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an example of a limited digestion of the transcription factor TFIIS from the yeast, *Saccharomyces cerevisiae*.

DETAILED DESCRIPTION OF THE INVENTION

Current limitations in the art of identifying stable domains of proteins for use in structure determination are overcome by use of this invention which provides a device and method for conducting limited proteolysis of proteins to identify protein domains useful for structure determination. In particular the device is designed to provide a high throughput of proteolytic digestion of proteins to identify domains and their boundaries, for use in protein structure determination, in a manner that is amenable to automation.

*The Device*Description of the Device as a Finished Product

The device comprises a panel of different proteases, wherein each type of protease is immobilized separately onto a solid support such as a well of a microtiter plate, and in increasing concentrations across the microtiter plate. A sort of protease digestion matrix is generated with different proteases along one axis and increasing concentrations along the other axis.

Different proteases can be used, both alkaline and acidic. Moreover, given the existence of a multitude of known proteases and the application of recombinant DNA technology to the study and production of protease analogs, the art has yet to develop completely. Many proteases are available and can be used in this device and procedure, for example: aminopeptidase M; bromelain; carboxypeptidase A, B and Y; chymopapain; chymotrypsin; clostripain; collagenase; elastase; endoproteinase Arg-C, Glu-C and LysC; Factor Xa; ficin; Gelatinase; kallikrein; metalloendopeptinidase; papain; pepsin; plasmin; plasminogen; peptidase; pronase; proteinase A; proteinase K; subtilisin; thermolysin; thrombin; and trypsin.

A solid support such as a microtiter plate is used. Each microtiter plate can have one or more proteases immobilized, at increasing concentrations across the plate. The larger the number of proteases with different specificity, the greater the procedural flexibility provided by one or more plates. In a preferred embodiment a single plate has six or more different immobilized proteases. Each protease is dispensed along a single row, with the concentration in the first well at 50 micrograms per millilitre and each subsequent well with a two-fold dilution. The device, after lyophilization, can be stable for a period of at least a week, at 4 °C.

Description of How to Make the Device

Immobilization of the enzyme on the solid support is readily accomplished by various methods which are known. Various methods are known for immobilization of enzymes (refer to, for

example, O. R. Zaborsky, "Immobilized Enzymes", C.R.C. Press, 1973; or "Immobilized Enzymes", edited by Ichiro Chihata, Kodansha, 1975; see also see I. Chibata, Editor, "Immobilized Enzymes", Halsted Press, John Wiley & Sons, Inc., New York, 1978, pp. 1-73.). They can roughly be classified into the following four groups: (1) physical or ionic
5 adsorption method; (2) covalent attachment method; (3) entrapment method; and (4) crosslinking method.

In a preferred embodiment, the device is generated by immobilizing the proteases by overnight incubation at room temperature.

The solid support generally can be either organic or inorganic. Examples of organic supports
10 include, among others, polyesters, such as poly(ethylene terephthalate); polyamides, such as nylon 6 and nylon 6.6; polyacrylates; polymethacrylates; polyacrylamides; poly(acrylic acid); poly(methacrylic acid); poly(galacturonic acid); poly(aspartic acid); ethylene-maleic anhydride copolymers; polyolefins, such as polyethylene, polypropylene, polybutene, and polybutadiene; polystyrene; poly(aminostyrene); poly(vinyl chloride); poly(vinyl alcohol); poly(vinylidene
15 chloride); cellulose and derivatives thereof; agarose gels; dextran gels and derivatives thereof; polysaccharides; polypeptides; collagen; and the like.

The inorganic supports can be classified as siliceous or nonsiliceous metal oxides. Examples of siliceous supports include, among others, glass, silica, wollastonite, bentonite, cordierite, and the like. Examples of nonsiliceous metal oxides include, among others, alumina, spinel, apatite,
20 nickel oxide, titania, zirconia, and the like.

In general, the support can be in any desired shape or form that is a continuous, shaped article such as a flat or curved sheet or a three-dimensional article such as a rectangular or cylindrical tube. As a practical matter, however, the support most often will be a microtiter plate or a similar shaped support.

For examples of procedures for immobilizing enzymes on inorganic supports, by way of illustration only, see U.S. Pat. No. 3,519,538 (which corresponds with French Patent No. 2,020,527), U.S. Pat. No. 3,556,945 (which corresponds with French Patent No. 2,001,336), and U.S. Pat. Nos. 3,666,627 and 3,802,997 (which correspond with French Patent No. 2,020,661).

- 5 In a preferred embodiment, the non-specific binding sites on the microtitre wells are blocked for a period of time (three hours) with an appropriate blocking solution containing 0.1 % beta-octylglucoside.

Description of How to Use the Device

- 10 The device can be used to treat a protein solution containing whole proteins or fragments thereof to limited proteolysis to generate stable domains of the proteins for further analysis.

- 15 The present invention provides proteolytically digested protein fragments substantially free of digesting protease by using proteases that are immobilized on a solid support such as a microtiter well. Coupling of the immobilized protease plates to a chromatographic step for separation of the protein fragments thereby generated, prepares the fragments for further analysis such as sequence determination or mass spectrometry. The design of the device allows for a high throughput of protein samples in addition to being particularly suitable for automation.

Automation

- 20 The loading and transference of the protein samples to the device and, following the reaction, to a detector such as a High Performance Liquid Chromatograph (HPLC) or mass spectrometer, can be performed by automated means known in the art.

Automated sampling and automated transfer and mixing of solutions and solvents is routine. Many commercial automated apparatuses are available and save considerable hours and days of

sample manipulation. For example, auto samplers for HPLC, gas chromatography and mass spectrometry are all commercially available. In addition, commercial solution handling devices are readily customised to suit the users needs. The employment of such automated handlers in conjunction with protease immobilized plates removes the steps of manipulating a protease solution and subsequent removal of unwanted protease reagent and protease fragments.

The use of the protease plates allows highly parallel domain mapping. The plates, with commercially available robotics, can be coupled with HPLC and mass spectrometry to make an integrated system for the rapid molecular identification of protein domains.

Advantages of the Present Invention

Advantages of the present invention include the primary factor that proteases and protease fragments do not substantially contaminate the proteolytically digested protein fragments.

Another advantage is the ability to re-use the treated plates

Another advantage is the reproducibility and high throughput of protease digestion.

A microtitre plate as used herein is a sample plate having one or more wells for receipt of a sample, the microtitre plate being suitable for use in an automated process. A plate well within a single microtitre plate can contain one or more proteases at a single concentration or differing concentrations or be a control blank. Due to the open layout of the microtiter wells, and the matrix of proteolytic enzymes, the design of the device permits a high throughput level of protein treatment that significantly diminishes the time required for an equivalent digestion procedure that is familiar in the art. Typically, such plates contain 96 wells, but higher volume plates such as 380 or more are also contemplated to function in the present invention.

Because many devices can be generated at the same time, there is an efficiency of production and

consistency of immobilized protease concentration that can be attained by this invention that is not currently available in the art. Some of the plates with an immobilized protease can be mass produced and stored for at least two weeks, at 4 °C, while maintaining activity. Plates can be manufactured as standards or as custom models as requested.

- 5 In contrast to autolytic proteases (and mixtures of proteases that can degrade due to one protease cleaving another protease, in solution), the ready use format of the present invention provides an immobilized product that: (1) is essentially unable to undergo autolytic cleavage; and (2) is essentially unable to degrade due to one protease cleaving another protease.

Description of the Kit

- 10 *A Kit for Conducting Limited Proteolysis Digestion of Proteins for Structure Determination Studies*

- In another embodiment, the present invention relates to a kit for conducting limited proteolysis digestion of proteins or protein fragments for structure determination analysis comprising at least one container including the above-described device. The proteolysis device can be presented in
15 a commercially packaged form, a packaged combination of one or more containers, devices, or the like, holding the necessary reagents and usually including written instructions describing the performance of the digestion procedure. Reagent systems of the present invention involve all possible configurations and compositions for performing the various digestion formats described herein.

- 20 In a preferred embodiment, the kit further comprises other containers comprising one or more of the following: wash reagents, dilution reagents, proteolysis stopping reagents and control proteins with known digestion patterns and instructions for use of the kit.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separated

containers. Such containers include glass or plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment or another. Such
5 containers will include a container which will accept the microtiter plate, a container which contains wash reagents and proteolysis stopping reagents (eg. acetic acid).

In an alternative embodiment, the kit will include the materials to prepare a device of the present invention, which would therefore, include proteolytic enzymes, blocking reagents and buffer materials. One skilled in the art will readily recognize that the proteolysis device of the present
10 invention can readily be incorporated into one of the established kit formats which are well known in the art.

EXAMPLE 1: A General Experimental Protocol for Immobilizing Proteases on
Microtiter Plates

In this example, four proteolytic enzymes are immobilized on a microtiter plate.

15 *Preparation of the Reagents*

The reagents were prepared as follows. A buffer solution of TBS, 50 mM Tris pH 8.0, 150 mM NaCl is prepared for use with the remaining solutions. A solution of TBS, 0.01% beta-octa-glucoside is prepared as a blocking buffer. The following proteases are prepared: Chymotrypsin, 0.5mg/ml (in TBS); Trypsin, 0.5mg/ml (in TBS); Papain, 0.5mg/ml (in TBS); and Proteinase K,
20 0.5mg/ml (in TBS). The protein to be digested is prepared at 65µg/ml (diluted in TBS).

Preparation of the Immobilized Protease Plate

In this example of device preparation, Nunclon surface 96 well microtiter plates are used. The proteases are immobilized onto the plates by aliquotting 45µl TBS into each well. Six wells are used for each protease to generate a series for each protease, each series operating at decreasing

concentrations across the wells by adding 5µl of protease stock solution to the first well, 2.5µl to the second well, etc. The serial dilution of the wells yield final concentrations of proteases of 50µg/ml, 25µg/ml, 5µg/ml, 2.5µg/ml, and 0.5µg/ml. The last well is left for protein only. The plate is covered and placed in a sealable bag with a wet paper towel. The resulting plate is incubated overnight at 4°C.

Blocking of an Immobilized Protease on a Microtiter Plate

The protease solution is removed from the wells by flicking the plate a few times while inverted. The wells are washed once with blocking buffer (100µl) and the excess solution is removed. 100µl of blocking buffer is added and incubate for 30 minutes at 4°C. The blocking buffer is removed and the wells are washed once (100µl) with TBS and the excess TBS is removed. The plates are now ready for use or can be lyophilized and stored at -20 °C until needed.

Digestion of an Protein by an Immobilized Protease

30µl of the protein solution of interest is added to each well. The resulting plate is then incubated at room temperature for 2-4 hours.

Analysis of Proteolytically Digested Protein

If the protein fragments are to be separated and analyzed by Mass Spectrometry, it is necessary to remove a sample of a proteolytically digested protein (5-10 µl) and stop the proteolysis by adding acetic acid to the sample until a final concentration of 1% is reached.

If the protein fragments are to be separated and analyzed by SDS PAGE Gel Electrophoresis, the proteolysis of protein is stopped by adding 5X protein loading dye to each well. The sample in each well is heated to approximately 90 °C for approximately 5 minutes. 20-25 µl of the sample is loaded onto a SDS PAGE gradient gel (5-18%), and run under electrophoresis followed by a Commassie stain to visualize the protein fragments.

EXAMPLE II: Comparison of Immobilized Protease Digestion to Solution Protease Digestion

The protease plates were used to digest three different proteins whose proteolytic digestion pattern in solution was already known. The pattern of digestion for the immobilized proteases mimicked the solution digestion pattern. Thus, we have shown that the immobilized proteases plates could be used to identify stable domains of several different proteins. Shown is an example of a limited digestion of the transcription factor TFIIS from the yeast, *Saccharomyces cerevisiae*.

EXAMPLE III: Results of Limited Proteolysis

Four different proteases, tyrypsin, chymotrypsin, papain and proteinase K (Sigma) were immobilized on plastic 96-well microtitre plates (Nuclon) in the following manner. The protease stocks were made 0.5 mg/ml in TBS (50mM Tris pH 8, 150 mM NaCl). A serial dilution of each protease was prepared to final concentrations of 50 µg/ml, 25 µg/ml, 5 µg/ml, 2.5 µg/ml and 0.5 µg/ml in TBS. 50 µl of each dilution was applied to different wells in a row of the microtitre plate. The plate with the arrayed protease dilutions was then incubated overnight at 4 ° C in a sealed bag containing a wet paper towel.

The protease solution was then removed and the wells washed with 100 µl of blocking buffer (TBS, 0.01% beta-octyl glucoside). The first wash was discarded and the non-specific binding sites on the microtitre wells were blocked with an additional 30 minute incubation at 4 ° C with an additional 100 µl of blocking buffer.

30 µl of a solution of yeast TFIIS (65 µg/ml) was incubated in each of the protease-coated wells for 2-4 hours at room temperature. 5 µl of the protein solution was then made to 2% Sodium dodecyl sulphate, 25% glycerol, 0.1M Tris-Hcl (pH 8.0) and resolved by gel electrophoresis. The results from a typical digestion (chymotrypsin) are shown in the previous example. The individual proteolytic products were purified either by reverse-phase liquid chromatography or by

elution from the gel slice and were analyzed by matrix-assisted desorption time-of-flight mass spectrometry. The fragments corresponded to known domains of the yeast TFIIS (Proc. Nat. Acad. Sci. U.S.A. 93: 10604-10608, 1996).

5 It is to be understood that the examples described above are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

I CLAIM:

1. A method for the preparation of proteolytically digested fragments of a protein, free of protease, for the determination of domains and the boundaries of the domains in said protein, the method comprises:
 - 5 (1) contacting a quantity of said protein with at least two concentrations of a protease under conditions which result in digested fragments of said protein, wherein each of said concentrations of protease is immobilized in a separate compartment; and
 - (2) separating said fragments from said immobilized protease.
- 10 2. A method according to claim 1 wherein said compartment is in an apparatus which comprises a plurality of compartments.
3. A method according to claim 2 wherein each compartment containing a quantity of said protease contains a concentration of said protease distinct from the concentration of said protease in every other
15 compartment.
4. A method according to claim 3 wherein at least three concentrations of protease are each in one of at least three different said compartments.
5. A method according to claim 4 wherein said plurality of
20 compartments forms a matrix in a linear format with at least three increasing concentrations of protease in said compartments.
6. A method according to anyone of claims 1-5 wherein said matrix contains more than one protease.

7. A method according to claim 6 wherein said matrix is in a two-dimensional format wherein: (i) distinct concentrations of each protease are aligned in increasing concentration, along a first axis or concentration axis; and (ii) different proteases are aligned along a second axis that is perpendicular to the first axis.

8. A method according to anyone of claims 1-7 wherein said protease is selected from the group consisting of aminopeptidase M; bromelain; carboxypeptidase A, B and Y; chymopapain; chymotrypsin; clostripain; collagenase; elastase; endoproteinase Arg-C, Glu-C and LysC; Factor Xa; ficin; Gelatinase; kallikrein; metalloendopeptinidase; papain; pepsin; plasmin; plasminogen; peptidase; pronase; proteinase A; proteinase K; subsilisin; thermolysin; thrombin; and trypsin.

9. A method according to claim 8 wherein the proteases are trypsin, chymotrypsin, proteinase K or papain.

10. A method according to claim 9 wherein the concentrations of each of the proteases along the first axis in each case is from about 50 μ g/mL to about 0.5 μ g/mL.

11. A method according to claim 10 wherein the concentration for each of the proteases is about 50 μ g/mL in one container of a concentration axis; about 25 μ g/mL in a second container of the concentration axis; about 5 μ g/mL in a third container of the concentration axis; about 2.5 μ g/mL in a fourth container of the

concentration axis; about 0.5 μ g/mL in a fifth container of the concentration axis; about 0 μ g/mL in a sixth container of the concentration axis.

12. A method according to any one of claims 1-11 wherein the surface
5 upon which said protease is immobilized has been treated with a blocker of surface interaction.

13. A method according to claim 12 wherein the blocker is selected from the group consisting of BSA and beta-octyl glucoside.

14. A method according to claim 1-13 wherein the apparatus may be
10 prepared and stored prior to use.

15. A method according to any one of claims 1 to 13 wherein the surface to which said protease is immobilized is selected from the group consisting of an organic support of material selected from the group consisting of polyesters, polyamides, polyacrylates, polymethacrylates,
15 polyacrylamides, poly(acrylic acid)m, poly(methacrylic acid); poly(galacturonic acid); poly(aspartic acid); ethylene-maleic anhydride copolymers; polyolefins; cellulose; cellulose derivatives; agarose gels; dextran gels and derivatives thereof, polysaccharides; polypeptides; collagen; and an inorganic support material selected from the group
20 consisting of siliceous and nonsiliceous metal oxides.

16. A method according to any one of claims 1 to 14 wherein said apparatus is a microtitre plate.

17. A method according to claim 16 wherein one of the conditions is

time for incubation and is between about 2 to 4 hours.

18. A method according to claim 17 wherein one of the conditions is temperature for a reaction and is room temperature.

19. A method according to anyone of claims 1 to 18 wherein only
5 limited digestion of said protein occurs.

20. A method for determining the boundaries of a proteolytically digested fragment of a protein which comprises the steps: (i) incubating a protein, according to the method of any one of claims 1-19 to yield protein fragments; (ii) isolation of fragment(s) of interest; (iii) determining the
10 mass of the isolated fragment(s); and (iv) matching the mass of the proteolytic fragment(s) to a protein amino acid sequence of the protein digested.

21. A method according to claim 20 wherein the determination of mass is by a method selected from the group consisting of nanospray
15 time-of-flight mass spectrometry and matrix-assisted time-of-flight mass spectrometry.

22. A method according to claim 21 wherein the protein fragment isolation is carried out by a technique selected from the group consisting of HPLC and SDS PAGE.

20 23. A method according to claim 20, 21, or 22 which is automated.

24. An apparatus for degradation of a protein, said apparatus comprising a plurality of compartments, each compartment containing a

quantity of a protease immobilized on a surface in each compartment wherein at least two compartments contain different concentrations of said protease.

25. An apparatus according to claim 24 wherein each compartment
5 containing a quantity of said protease contains a concentration of said protease distinct from the concentration of said protease in every other compartment.

26. An apparatus according to claim 25 wherein at least three concentrations of protease are in said compartments.

10 27. An apparatus according to claim 26 wherein said plurality of compartments forms a matrix in a linear format with at least three increasing concentrations of protease in said compartments.

28. An apparatus according to anyone of claims 24-27 wherein said matrix contains more than one protease.

15 29. An apparatus according to claim 28 wherein said matrix is in a two-dimensional format wherein: (i) distinct concentrations of each protease are aligned in increasing concentration, along a first axis or concentration axis; and (ii) different proteases are aligned along a second axis that is perpendicular to the first axis.

20 30. An apparatus according to anyone of claims 24-29 wherein said protease is selected from the group consisting of aminopeptidase M; bromelain; carboxypeptidase A, B and Y; chymopapain; chymotrypsin; clostripain; collagenase; elastase; endoproteinase Arg-C, Glu-C and LysC;

Factor Xa; ficin; Gelatinase; kallikrein; metalloendopeptinidase; papain; pepsin; plasmin; plasminogen; peptidase; pronase; proteinase A; proteinase K; subsilisin; thermolysin; thrombin; and trypsin.

31. An apparatus according to claim 30 wherein the proteases are
5 trypsin, chymotrypsin, proteinase K or papain.

32. An apparatus according to claim 31 wherein the concentrations of each of the proteases along the first axis in each case is from about 50 μ g/mL to about 0.5 μ g/mL.

33. An apparatus according to claim 32 wherein the concentration for
10 each of the proteases is about 50 μ g/mL in one container of a concentration axis; about 25 μ g/mL in a second container of the concentration axis; about 5 μ g/mL in a third container of the concentration axis; about 2.5 μ g/mL in a fourth container of the concentration axis; about 0.5 μ g/mL in a fifth container of the
15 concentration axis; about 0 μ g/mL in a sixth container of the concentration axis.

34. An apparatus according to any one of claims 24-33 wherein the surface upon which said protease is immobilized has been treated with a blocker of surface interaction.

20 35. An apparatus according to claim 34 wherein the blocker is selected from the group consisting of BSA and beta-octyl glucoside.

36. An apparatus according to any one of claims 24 to 33 wherein the

surface to which said protease is immobilized is selected from the group consisting of an organic support of material selected from the group consisting of polyesters, polyamides, polyacrylates, polymethacrylates, polyacrylamides, poly(acrylic acid)m, poly(methacrylic acid);
5 poly(galacturonic acid); poly(aspartic acid); ethylene-maleic anhydride copolymers; polyolefins; cellulose; cellulose derivatives; agarose gels; dextran gels and derivatives thereof, polysaccharides; polypeptides; collagen; and an inorganic support material selected from the group consisting of siliceous and nonsiliceous metal oxides.

10 37. An apparatus according to claim 24-36 wherein the apparatus may be prepared and stored prior to use.

38. An apparatus according to any one of claims 24 to 37 wherein said apparatus is a microtitre plate.

FIGURE 1

PROTEOLYSIS PLATE

SOLUTION

