Title: METHODS FOR FOLDING PROTEINS AND REDUCING PROTEIN AGGREGATION

Abstract: The invention relates to methods, kits and consumables for folding protein and for controlling protein folding and aggregation in which denatured protein is contacted with a folding control compound, suitably a polymer such as sugar polymer, that maintains the protein essentially in a non-native, non-aggregated state; protein folding is carried out in a controlled manner by degrading the folding control compound(s), thereby enabling protein folding to take place. The present invention relates to methods and kits for removing folding control compounds from protein solutions.
Methods for folding proteins and reducing protein aggregation

5 Technical Field

The present invention relates to methods, kits, consumables and reagents for protein folding, in which inter-protein and intra-protein interactions are controlled, using folding control compounds, to achieve efficient protein folding with reduced aggregation. The present invention also relates to methods for removing folding control compounds from protein solutions.

Background to the invention

15 The biological function of a protein is dependent on its three dimensional structure. Proteins are formed as linear chains of amino acids, termed polypeptides. In vivo, in the appropriate conditions within the cell, the linear polypeptide chain is folded, a process which may be assisted by proteins called chaperones. A mature folded protein has an active three dimensional conformation, known as the native structure. The structure depends on weak forces such as hydrogen bonding, electrostatic and hydrophobic interactions. These forces are affected by the protein environment, so changes in the environment may cause structural disruption resulting in denaturation of the protein and loss of function.

20 The production of proteins by genetic engineering often results in the accumulation of non-active protein aggregates as inclusion bodies. After isolation and purification from the host cells, proteins or inclusion bodies have to be completely unfolded (denatured) and subsequently refolded (renatured) so that the proteins regain their native structure and bioactivity.
Traditional protein folding methods involve denaturant dilution or column-based approaches. Denatured protein is commonly refolded by diluting the denaturant away. This induces a hydrophobic collapse of the protein molecule and in doing so the protein shields its hydrophobic patches in the core of the molecule. Unfortunately, on hydrophobic collapse, proteins do not always form the native bioactive conformation; two competing reactions occur: refolding and aggregation. It is suggested that the driver for protein aggregation is hydrophobic amino acid residues exposed at the surface. Aggregation is undesirable and reduces the yield of functional, native protein. Refolding is known as a first order reaction (Rate = $K \cdot [\text{prot}]$), but the aggregation reaction is favoured over refolding as it is a higher order reaction ($n$) (Rate = $K' \cdot [\text{prot}]^n$). The proportion of protein refolding:protein aggregation is strongly dependent on the protein concentration. At process scale this concentration dependency can result in increased aggregation, and thus reduced refolding yield. This can be due to imperfect mixing patterns in the protein solution. Processing large protein molecules is particularly difficult as they diffuse more slowly than the smaller denaturant molecules, thus creating micro-environments containing high localised protein concentrations and low denaturant concentration, that is, an environment that favours protein aggregation over protein refolding.

For proteins with disulphide bonds the native protein often needs to be “matured”; during maturation, non-aggregated monomeric protein molecules with non-native disulphide bonds are created initially; then, using a protein specific redox couple, the disulphide bonds shuffle and the protein matures to a functional protein molecule with native disulphide bonds.

Refolding processes usually involve dispersing the denatured protein molecules in a buffer in the presence of “refolding aids” to enhance renaturation. Folding aids usually increase the solubility of the folding intermediates and/or change the relative reaction rates of the folding and aggregation reactions. Polyethylene glycol and various sugars and detergents have been employed as refolding aids.
Cyclodextrins (CDs) have been reported to be useful in stabilisation, solubilisation and affinity purification of certain enzymes, but both the nature of the interactions between CDs and proteins, and their effect on bioactivity remain unclear. Cyclodextrins have been used as artificial chaperones to aid protein refolding in both detergent-free (Sharma et al, EP 0 871 851, US 4,659,696) and detergent-containing refolding environments (Gellman & Rozema, US 5,563,057). Cyclodextrins are cyclic oligosaccharides composed of multiple glucose residues. They are classified according to the number of sugar residues within the ring structure, α-cyclodextrin has 6 glucose residues, β-cyclodextrin has 7 glucose residues and γ-cyclodextrin has 8 glucose residues. Cyclodextrins can be modified by derivatisation to produce derivatives.

The inner cavity of cyclodextrins is hydrophobic whereas the outer surface is hydrophilic. The hydrophobic interior is capable of encapsulating poorly soluble drugs. The hydrophilic exterior assists in solubilisation, so cyclodextrins are useful adjuncts in pharmaceutical formulation.

EP 0 094 157 & US 4,659,696 (Hirai et al) describe the use of cyclodextrin derivatives in pharmaceutical compositions consisting essentially of a physical mixture of a hydrophilic, physiologically active (folded, native) peptide and a cyclodextrin derivative, the composition being a uniform mixture in dosage form.

EP 0 437 678 B1, US 5,730,969 and US 5,997,856 (Hora) describe methods for the solubilisation and/or stabilisation of polypeptides, especially proteins, using specified cyclodextrin derivatives: hydroxypropyl, hydroxyethyl, glucosyl, maltosyl, and maltotriosyl derivatives of β- and γ-cyclodextrin; the hydroxypropyl-β-cyclodextrin derivative being preferred. Also disclosed are aqueous and lyophilised compositions comprising a polypeptide, optionally a protein, and the above specified cyclodextrin derivatives.
EP 0 871 651 & US 5,728,804 (Sharma et al) are concerned with a method for renaturing an unfolded or aggregated protein in a detergent-free aqueous medium with an amount of a cyclodextrin effective to renature said unfolded or aggregated protein. In this instance, the protein is present at a low concentration selected to minimise aggregation, preferably at around 0.05 mg/ml, and spontaneously refolds in a refolding buffer containing cyclodextrin. After refolding the cyclodextrin is removed by dialysis.

US 5,563,057 (Gellman & Rozema) describes a method for refolding an enzyme from a misfolded configuration to a second native active configuration by adding a detergent having a linear alkyl non-polar portion, e.g. CTAB and Triton®-X 100 (Octylxynol-9), to misfolded enzyme to form an enzyme-detergent complex, which is then contacted with a cyclodextrin to allow the enzyme to assume a second active conformation.

Thus when a cyclodextrin is used as a protein folding aid, it is removed from the protein folding reaction mixture during refolding by “capture” by appropriate detergents (as described by Gellman & Rozema, ibid), or after refolding by either dialysis (Sharma et al, ibid.), or dilution.

US 5,342,633 (Cully et al) describes a method for reduction of cholesterol in egg yolk, which involves complexing the cholesterol with a cyclodextrin; after removal of the cholesterol-cyclodextrin complexes, contaminant cyclodextrin is removed from the egg yolk by treatment with water and/or an alcohol and/or by amylase digestion.

US 5,532,005 (Hedges et al) describes treatment of a food system (such as eggs, dairy, meat, fruit juices, coffee and tea, starch hydrolysates and protein hydrolysates) which contains residual cyclodextrin with both CGTase and an amylase at a temperature of 40°C to 80°C, a pH of 4 to 6 for a time of 1 to 48 hours to hydrolyse residual cyclodextrin.
Hinrichs et al (2001). International Journal of Pharmaceutics, 215, 163-174, describes the use of non-derivatised inulins, inulin SC 95 (DPn/DPw = 5.5/6.0), inulin RS (DPn/DPw = 14.2/19.4), and inulin EXL 608 (DPn/DPw 23.0/26.2) to protect alkaline phosphatase from degradation during freeze drying and subsequent storage of the dried protein.

WO 96/41870 (Gombac et al) describe frozen, dried or lyophilised hydrosoluble collagenase compositions containing isomalt and inulin to stabilise the collagenase.

To date, inulins have not been reported to be useful as protein folding aids. Inulins are D-fructans, generally consisting of chains of polyfructose in which the fructose units are connected to each other mostly or exclusively by beta (2-1) linkages. Inulin occurs in nature, in general, as a polydisperse mixture of polyfructose chains most of which are ending in one glucosyl unit. Inulin can be obtained from bacterial synthesises, extracted from plants or can be made in vitro by enzymatic synthesis starting from sucrose. Inulin produced by bacteria is more branched than inulin from plant origin and commonly has a higher molecular weight (ranging from about 2,000 up to about 20,000,000), whereas inulin from plant origin is generally composed of linear or slightly branched polyfructose chains, or mixtures thereof, with a molecular weight commonly ranging from about 600 to about 20,000.

Inulin can be represented, depending from the terminal carbohydrate unit, by the general formulae GF.n or F.n, wherein G represents a glucosyl unit, F a fructosyl unit, and n is an integer representing the number of fructosyl units linked to each other in the carbohydrate chain. The number of saccharide units (fructose and glucose units) in one inulin molecule, i.e. the value of n in the formulae above, is referred to as the degree of polymerisation, represented by (DP). Often, the parameter (number) average degree of polymerisation, represented by (DP), is used too, which is the value corresponding to the total number of saccharide units (G and F units) in a given inulin composition divided
by the total number of inulin molecules present in said inulin composition, without taking into account the possibly present monosaccharides glucose (G) and fructose (F), and the disaccharide sucrose (GF). The average degree of polymerisation (DP) can be determined, for example, by the method described by L. De Leenheer (Starch, 46 (5), 193-196, (1994), and Carbohydrates as Organic Raw Materials, Vol. III, p. 87-92, (1996)).

Inulin is commonly prepared from plant sources, mainly from roots of Chicory (Cichorium intybus) and from tubers of Jerusalem artichoke (Helianthus tuberosus), in which inulin can be present in concentrations of about 10 to 20% w/w on fresh plant material. Inulin from plant origin is usually a polydisperse mixture of linear and slightly branched polysaccharide chains with a degree of polymerisation (DP) ranging from 2 to about 100. In accordance with known techniques, inulin can be readily extracted from said plant parts, purified and optionally fractionated to remove impurities, mono- and disaccharides and undesired oligosaccharides, in order to provide various grades of inulin, e.g. as described in EP 0 769 026 and EP 0 670 850.

Inulin is commercially available, typically with a (DP) ranging from about 6 to about 40. Inulin from chicory is for example available as RAFTILINE® from Orafti (Tienen, Belgium) in various grades. Typical RAFTILINE® grades include RAFTILINE® ST (with a (DP) of about 10 and containing in total up to about 8% by weight glucose, fructose and sucrose), RAFTILINE® LS (with a (DP) of about 10 but containing in total less than 1% by weight glucose, fructose and sucrose), and RAFTILINE®.RTM. HP (with a (DP) of at least 23, commonly with a (DP) of about 25, and virtually free of glucose, fructose and sucrose). Inutec®N25, a non-derivatised inulin with a DP of 25, is available from Orafti.

Inulins with a lower degree of polymerisation, usually defined as a (DP) <10, are commonly named inulo-oligosaccharides, fructo-oligosaccharides or oligofructose. Oligofructose can be conventionally obtained by partial (preferably enzymatic) hydrolysis of inulin and can also be obtained by
enzymatic in vitro synthesis from sucrose according to techniques which are well-known in the art. Several grades of oligofructose are commercially available, for example as RAFTILOSE® from Orafti, (Tienen, Belgium), e.g. RAFTILOSE® P95 with a mean content of about 95% by weight of oligofructose with a degree of polymerisation (DP) ranging from 2 to 7 and containing about 5% by weight in total of glucose, fructose and sucrose.

Various inulin derivatives and methods for the preparation of inulin derivatives are described in US 6,534,647 (Stevens et al), the entire contents of which are incorporated herein by reference.

Inulins derivatised with hydrophobic alkyl chains on the polyfructose backbone are commercially available, for example Inutec® SP1 (SP1) from Orafti (Tienen, Belgium).

There is a need for methods for the efficient preparation of correctly folded, non-aggregated, active protein, particularly proteins produced using recombinant techniques. Control of folding, aggregation and stability of proteins during processing and on storage is a recognised problem in many industries, in particular the pharmaceutical and biotechnology industries. Difficulties encountered with proteins may make manufacture of proteins difficult, result in low yields and render processes uneconomic. Protein folding and stabilising aids, methods, consumables, reagents and kits that permit control of protein refolding, modulate protein aggregation and promote protein stability are commercially important.

Problems addressed by the present invention include reducing protein aggregation and achieving control of protein refolding, in particular maintaining protein in a stable non-native state during protein folding, then restoring the original protein properties by folding the protein in a controlled manner to reduce aggregation and thereby increase the yield of soluble intermediates or native protein.
Disclosure of invention

The present invention provides a method for folding protein comprising:
(a) in aqueous solution, contacting denatured protein with one or more folding
control compounds(s), and
(b) degradation of the folding control compound(s).

Also provided is a method for controlling protein folding comprising:
(a) in aqueous solution, contacting denatured protein with one or more folding
control compound(s), and
(b) controlled degradation of the folding control compound(s).

Further provided is a method for folding protein comprising:
(a) providing an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more folding control compound(s) present at a concentration sufficient to maintain protein in denatured conformation, and
(c) degradation of the folding control compound(s), to allow the protein to fold.

According to the methods of the present invention, denatured protein is contacted with a folding control compound or compounds that can maintain the protein essentially in a non-native, non-aggregated state; protein folding is carried out in a controlled manner by degrading the folding control compound(s) and thereby affecting the protein structure. Protein folding can be carried out at relatively high protein concentration and preferably in the absence of denaturant(s).

The term “protein” as used herein encompasses proteins, peptides, polypeptides and oligopeptides. Proteins may be synthetic or naturally occurring, and may be obtained by chemical synthesis, or by recombinant or non-recombinant methods.
The denatured protein in aqueous solution can be fully denatured, or partially denatured or renatured such that the protein is in non-native form as unfolded protein and/or partially folded refolding intermediate(s). The aqueous solution of denatured protein may contain one or more of these forms. Some protein may also be present in the solution of denatured protein in the form of contaminating aggregates and/or inclusion bodies.

The present invention also provides a method for folding protein comprising:
(a) using a chaotrope, detergent, and/or reducing agent to denature protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more folding control compound(s) present at a concentration sufficient to maintain protein in denatured conformation,
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the folding control compound(s), to allow the protein to fold.

Suitable chaotropes for use in methods of the invention to denature protein and/or dissolve aggregated protein and/or inclusion bodies include guanidine hydrochloride (e.g. 6M), and urea (e.g. 8M).

Suitable reducing agents for use in methods of the invention to denature protein and/or dissolve aggregated protein and/or inclusion bodies include dithiothreithiol, dithioerythritol, beta-mercaptoethanol or Tris (2-carboxyethyl) phosphine hydrochloride (TCEP.HCl), preferably at a concentration in the range of from 1 to 50 mM.

Suitable detergents for use in methods of the invention to denature protein and/or dissolve aggregated protein and/or inclusion bodies include an anionic detergent (e.g. lauroyl sarcosine, Sodium dodecyl sulfate (SDS)), a cationic detergent (e.g. cetyl trimethylammonium bromide (CTAB)), a non-ionic
detergent (e.g. Triton®-X 100, Tween® 60, dodecylmaltoside and/or a zwitterionic detergent (e.g. CHAPS).

The concentration of chaotrope, detergent and/or reducing agent can be reduced by dilution or buffer exchange through dialysis, diafiltration, filtration, chromatographic methods, such as gel permeation, size exclusion, chromatography, ion exchange chromatography and/or affinity chromatography. Ideally the concentration of chaotrope, detergent and/or reducing agent is reduced to a level that would support the bioactive conformation, in the absence of the folding control compound(s) or any other additive, e.g. typically below 1M urea or below 0.5 M guanidine hydrochloride. The folding control compound or compounds(s) is/are present at a concentration sufficient to maintain protein in denatured conformation, even when the concentration of chaotrope, detergent and/or reducing agent is reduced to a level which would, in the absence of the folding control compound, normally result in refolding of the protein to some extent.

Protein folding encompasses both folding and refolding. The protein may refold to form a partially folded protein folding intermediate and/or a folded functional protein.

Protein folding conditions (buffer, ionic strength, pH, temperature, redox potential, presence of metal ions or co-factors) are highly protein specific and will be known or can be readily derived by those skilled in the art. Suitable temperatures for performing method of the invention will generally fall in range of from about 0°C to about 45°C, preferably from about 4°C to about 42°C, further preferably from about 4°C to about 37°C, yet further preferably from about 4°C to about 30°C, more preferably from about 4°C to about 25°C. The duration of an incubation step is typically 72 hours or less, preferably 48 hours or less, more preferably 24 hours or less, e.g. 1, 2, 4, 8, 12, or 15 hours.
Methods according to the present invention enhance refolding yield in a range of conditions previously known or and may extend the range of conditions that support protein refolding. The reaction conditions, protein concentration and the concentration of the folding control compound are such that initially the protein is essentially maintained in a non-aggregated non-native or partially folded intermediate state; a small amount, preferably less than 5% of the protein may be present in the form of contaminating aggregates and/or inclusion bodies. Removal of the folding control compound by degradation permits refolding of the protein, in a controlled manner, with reduced aggregation.

A folding control compound is a compound which, at an appropriate concentration and in appropriate conditions, can maintain a protein in solution in a non-native or partially-folded soluble state. A folding control compound can be any compound that is capable of shielding hydrophobic amino acid side chains or modifying the net protein charge or hydrogen bonding characteristics and thus altering the protein structure. The folding control compound is preferably a polymer. The term "polymer" encompasses polymeric and oligomeric molecules. A folding control compound is preferably a sugar polymer (oligosaccharide) or a derivative thereof and can be a linear or non-linear amphipathic sugar polymer or a derivative thereof. Sugar polymers employed in method of the invention may comprise one or more sugar selected from the group comprising glucose, fructose, mannose and/or galactose, which are the four most common simple (monomer) sugar units. The sugar polymer can be a dextran, cellulose, amylose, starch, pullulan, mannann, chitin, chitosan, inulin, levan, xylan, cyclodextrin, cycloamylose or a derivative thereof. Suitable sugar polymers derivatives are disclosed in US 5,202,433 and 5,204,457, the entire contents of which are incorporated herein by reference. Amphipathic sugar polymers are capable of hydrophobic interaction with native and/or denatured protein. Derivatisation of the sugar polymer with appropriate substituents enhances the amphipathic nature and strength of interaction with the protein. Suitable substituents include linear alkyl chains, branched alkyl
chains, double or triple bonded hydrocarbons, aromatic groups, fatty acids or polyols. More than one substituent per carbohydrate molecule can be present.

In a preferred embodiment of the invention, the folding control compound is a cyclic sugar polymer, such as glucosan e.g. a cyclodextrin or derivative thereof, most preferably an α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin, or a derivative thereof. The terms "cyclodextrin" and "cyclodextrin(s)" as used herein include cyclodextrins such as α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin and derivatives thereof.

When the folding control compound is a cyclodextrin or a derivative thereof, the initial concentration used in methods of the invention to maintain protein in a denatured conformation, i.e. in a non-native or partially folded soluble state prior to degradation and folding of the protein, is preferably from 100 to 10,000 times the molar concentration of protein present, most preferably from 100 to 5,000 times the molar concentration of protein present, yet more preferably from 100 to 2,000 times the molar concentration of protein present, further preferably from 500 to 1,500 times the molar concentration of protein present, most preferably at about 1,000 times the molar concentration of protein present.

The folding control compound can be a helical sugar polymer such as a fructosan, e.g. an inulin or a derivative thereof, suitably with a degree of polymerisation of from about 3 to about 500, about 3 to about 250, about 3 to about 100, about 3 to about 50, about 10 to about 50, about 15 to about 40, or from about 20 to about 30. An inulin derivative for use in a method of the invention can be substituted with one or more type(s) of non-polar hydrocarbyl group, for example a linear alkyl substituent or a branched alkyl substituent.

When the folding control compound is an inulin or a derivative thereof, the initial concentration used in methods of the invention to maintain protein in a denatured conformation, i.e. in a non-native or partially folded soluble state prior to degradation and folding of the protein, is in the range of from about 1 to about
1000 times the molar concentration of protein, preferably from about 1 to about
100 times the molar concentration of protein.

One, or more (i.e. a mixture of), folding control compound(s) may be used in
methods of the invention.

Degradation of the folding control compound(s) is preferably by enzymic
digestion and/or by chemical degradation. Alternatively or additionally,
degradation can be achieved using electromagnetic radiation, shear stress, or
heat. When the folding control compound is a sugar polymer or a derivative
thereof, degradation is preferably by enzymic digestion, e.g. using carbohydrate
degrading enzymes such as amylase, glucosyl transferase, cellulases, or
debranching enzymes. For cyclodextrin(s), degradation is preferably by
glucosyltransferase and/or amylase digestion. For inulins, degradation is
preferably by exo-inulinase and/or endo-inulinase digestion. Degradation
means reduction of the concentration of the reagent being degraded, and can
be a partial degradation or essentially complete degradation of the reagent.

In methods of the invention, a disulphide shuffling agent may be present during
degradation of the folding control compound. Suitable disulphide shuffling
agents for use in methods of the invention include reduced and oxidised
glutathione, cysteine, cystine and disulphide isomerases, preferably at a
concentration in the range of from 10μM to 10mM. Disulphide isomerases are
preferably used at a concentration of from 10μM to 10 mM.

Methods according to the present invention permit folding/refolding of denatured
proteins in solution so that properly folded, native conformation protein in
solution can be efficiently recovered. Methods of the invention can be applied
to virtually any protein, especially after solubilisation and/or denaturation of
insoluble protein aggregates, inclusion bodies, or abnormal soluble aggregates.
In preferred embodiments one or more cyclodextrin or derivative thereof is used as the folding control compound, accordingly, the invention provides a method for folding protein comprising:
(a) in aqueous solution, contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, and
(b) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

Also provided is a method for controlling protein folding comprising:
(a) in aqueous solution, contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, and
(b) controlled degradation of the cyclodextrin(s) and/or derivative(s) thereof.

Further provided is a method for folding protein comprising:
(a) providing an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present in solution at a concentration sufficient to maintain protein in denatured conformation, and
(c) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

Yet further provided is method for refolding protein comprising:
(a) using a chaotrope, detergent, and/or reducing agent to denature protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present at a concentration sufficient to maintain protein in denatured conformation,
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.
Additionally the invention provides a method for refolding protein comprising:
(a) providing an aqueous solution comprising denatured protein using a chaotrope, detergent and/or reducing agent to denature protein and/or dissolve aggregated protein and inclusion bodies,
(b) providing to the aqueous protein solution of (a) one or more cyclodextrin(s) and/or derivative(s) thereof, at a concentration sufficient to maintain the protein essentially in non-native conformation, preferably by buffer exchange to a solution containing one or more cyclodextrin(s) and/or derivative(s) thereof.
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the one or more cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

Suitable chaotropes, reducing agents and detergents are described above.

Where the method involves reducing the concentration of chaotrope, detergent and/or reducing agent, this can be achieved by dilution, or buffer exchange through dialysis, diafiltration, filtration, and/or a chromatographic method, such as gel permeation, size exclusion, chromatography, ion exchange chromatography and/or affinity chromatography.

Protein aggregation during protein folding can be controlled by controlling the rate of degradation of the cyclodextrin molecules(s), thus the invention provides a method for controlling protein aggregation during protein folding comprising:
(a) providing an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present in aqueous solution at a concentration sufficient to maintain the protein in non-aggregated form, and
(c) degradation of said one or more cyclodextrin(s) and/or derivative(s) thereof, in a controlled manner to induce protein folding.

In method of the invention, cyclodextrin(s) and/or derivative(s) thereof are present in solution initially at a concentration sufficient to prevent complete
renaturation and thus maintain protein essentially in a denatured, non-native, unfolded or partly folded intermediate conformation. An initial concentration of the one or more cyclodextrin(s) or derivative(s) thereof sufficient to maintain protein essentially in denatured conformation is preferably from about 1 to about 200 mg/ml, more preferably from about 1 to about 100 mg/ml, yet more preferably from about 10 to about 50 mg/ml, further preferably from about 10 to about 30 mg/ml, most preferably about 20 mg/ml. Such concentrations will stabilise the protein in a non-native conformation and maintain the protein essentially in a non-aggregated form, although a small amount of aggregated protein may be present. Without wishing to be bound by theory, it is believed that as the concentration of the cyclodextrin is decreased by degradation, hydrophobic patches on the protein are gradually exposed and folding can take place to form the native conformation.

Suitably, degradation of the cyclodextrin(s) and/or derivative(s) thereof is by one or more of the following methods: enzymic digestion, chemical degradation, electromagnetic radiation, shear stress and/or heat, enzymic digestion and/or chemical degradation are preferred, particularly preferred is enzymic digestion by glucosyltransferase and/or amylase digestion. A disulphide shuffling such as a reduced or oxidised glutathione, cysteine, cystine and a disulphide isomerase may be present during the degradation reaction.

Using methods of the invention, the rate of degradation of the folding control compound can be controlled in order to control protein folding or refolding. For example, the rate of glucosyltransferase or amylase digestion of cyclodextrin can be controlled by selecting enzymes with different activity, adjusting the enzyme concentration, solution pH, temperature, ionic strength/composition, redox potential and/or addition of enzyme cofactors. Suitable reaction conditions can be readily determined by those skilled in the art. The benefits of controlling protein folding are the ability to minimise the local concentration of aggregation prone folding intermediates or non-native protein molecules.
In prior art methods, refolding is carried out at low protein concentrations, (e.g. for lysozyme usually less than 0.10 mg/ml). Sharma et al describe methods for renaturation of unfolded or aggregated CAB protein that involve contacting the protein in detergent free medium with an amount of cyclodextrin effective to renature the unfolded or aggregated protein. In these methods the protein concentrations are low, selected to minimise aggregation, preferably at around 0.05 mg/ml. Refolding occurs in the presence of cyclodextrin and it is suggested that during refolding the relatively polar cyclodextrin molecules that are weakly bound to hydrophobic sites in the folding intermediates are gradually displaced as the interior of the protein becomes increasingly non-polar during refolding. The cyclodextrins are not removed from the solution to achieve refolding, indeed Sharma states that "Since the cyclodextrins can inhibit aggregation without interfering with protein refolding, they are highly effective protein folding agents". Sharma also advises that that cyclodextrins can be easily separated, if desired, from the refolded protein by dialysis or gel filtration. Thus in methods according to Sharma, when cyclodextrin is removed, it is removed after refolding of the protein.

However, methods, reagents, consumables and kits of the invention, enable protein folding over a range of protein concentrations, at higher protein concentrations than traditional methods and with reduced aggregation. Protein concentrations at which methods of the invention can be performed are generally in the range of from 0.001 to 0.5 mg/ml, preferably in the range of 0.001 to 0.25 mg/ml, more preferably in the range of from 0.001 to 0.15 mg/ml. The protein concentration chosen will depend on factors such as the size of the protein. Although several smaller proteins have been successfully refolded at higher protein concentrations, generally a lower protein concentration is needed for folding large proteins like antibodies. The advantage of using high protein concentrations is reduced processing volume, resulting in easier purification, reduced reagent usage and reduced capital expenditure (smaller equipment size).
The present invention also provides a method for folding protein comprising:
(a) providing an aqueous solution comprising protein in non-native conformation
and an inulin or inulin derivative or a mixture of inulin(s) and/or inulin
derivative(s),
and,
(b) degradation of the inulin(s) and/or inulin derivative(s), to permit folding of the
protein.

Also provided is a method for controlling protein folding comprising:
(a) providing an aqueous solution comprising protein in non-native conformation
and an inulin or inulin derivative or a mixture of inulin(s) and/or inulin
derivative(s), and,
(b) controlled degradation of the inulin(s) and/or inulin derivative(s), to permit
folding of the protein.

The inulin or inulin derivative can be gradually degraded, in a controlled
manner, to permit folding with reduced aggregation and provide a higher yield of
native, active protein.

The concentration of the inulin or inulin derivative or the a mixture of inulin(s)
and/or inulin derivative(s) using in methods for folding protein depend on
various factors such as the temperature, solution conditions and the protein
itself. Suitable concentrations of the inulin or an inulin derivative or a mixture of
inulin(s) and/or inulin derivative(s) for folding protein can be readily determined
by those skilled in the art. In methods for folding protein, the inulin or inulin
derivative or mixture of inulin(s) and/or inulin derivative(s) is suitably present
initially in the folding reaction solution (i.e. prior to degradation) at a low to
medium concentration, e.g. at a concentration of from 0.002 mg/ml to 20 mg/ml,
preferably of from 0.01 to 5mg/ml.

Degradation of the inulin(s) and/or inulin derivative(s) can be performed by one
or more of the following methods: enzymic digestion, chemical degradation
electromagnetic radiation, shear stress or heat. In particularly preferred methods degradation of the inulin(s) and/or derivatives is performed by enzymic digestion and/or chemical degradation, most preferably by enzymic digestion, which can be performed using one or more exo-inulinase(s) and/or endo-inulinase(s), such as the exo-inulinase Fructan β-fructosidase E.C.3.2.1.80 and/or the endo-inulinase E.C.3.2.1.7.


Using methods of the invention, the rate of degradation of the inulin(s) and/or inulin derivative(s) can be controlled, for example by selecting enzymes with different activity, adjusting the enzyme concentration, solution pH, temperature, ionic strength/composition, redox potential and/or addition of enzyme cofactors. Suitable reaction conditions can be readily determined by those skilled in the art. The benefits of controlling protein folding are the ability to minimise the local concentration of aggregation prone folding intermediates or non-native protein molecules.

Additionally, removal of the inulin(s) and/or derivative(s) thereof can be performed by dilution, buffer exchange through dialysis, diafiltration, filtration, precipitation and/or a chromatographic method suitable chromatographic
methods include gel permeation, size exclusion chromatography, ion exchange chromatography, or affinity chromatography.

In folding methods of the invention in which one or more inulins (s) or derivative(s) thereof are employed, one or more of a chitin, chitosan, cyclodextrin, cycloamylose, or a derivative thereof, a hydrophobic resin and/or a hydrophobic gel can be included in the folding solution, and can be included at the start of the protein folding reaction, or included in solution during the protein folding reaction during incubation, i.e. after an initial incubation period. Such methods may further comprise removal of the one or more of the chitin, chitosan, cyclodextrin, cycloamylose or a derivative thereof, a hydrophobic resin and/or a hydrophobic gel from the solution, either during or after the folding reaction. The terms “cyclodextrin” and “cyclodextrin(s)” as used herein include cyclodextrins such as α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin and derivatives thereof. One or more (i.e. a mixture of), cyclodextrin(s) may be used in certain methods of the invention. Various chitins, chitosans and cycloamyloses are suitable for use in protein folding methods of the invention; chitins are commercially available for many sources, e.g. Sigma Aldrich. One or more chitin and/or one or more chitosan and/or one or more cycloamylose, or a derivative or derivatives thereof may be used in protein folding methods of the invention. Suitable hydrophobic resins (beads) and gels for use in protein folding methods of the invention include those with a hydrophilic outer surface and a pore size sufficient to exclude the protein. The pore/core is hydrophobic and capable of binding molecules such as detergents to partially or essentially completely remove the detergent. Commercially available sources include Bio-Beads SM Adsorbents (Bio-Rad) and Extractic-Gel D Detergent Removing Gel (Pierce, Perbio in UK).

The invention also provides protein folding methods in which, in addition to an inulin(s) and/or a derivative(s) thereof, a cyclodextrin, a cyclodextrin derivative or a mixture of cyclodextrin(s) and/or cyclodextrin derivative(s) can included in the protein folding solution. The cyclodextrin(s) is preferably introduced into the
protein folding solution during the folding process, e.g. after an initial incubation period. Protein folding methods of the invention may involve a multi-step incubation, e.g. a two step incubation, in which the protein is initially incubated with one or more inulin and/or inulin derivative (e.g. for 30 minutes, 1, 2, 4, 6, 8 or 12 hours) and then one or more cyclodextrin and/or cyclodextrin derivative can be introduced into the solution and the incubation continued. In methods of the invention it is believed that the cyclodextrin will weaken the interaction between the protein and inulin and/or inulin derivatives and thus help the protein to refold. Such methods may further comprise removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) from the solution; the cyclodextrin(s) can be removed from the solution during and/or after protein folding. Removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) from the solution is preferably by degradation, e.g. by one or more of the following methods: chemical degradation, enzymic degradation, electromagnetic radiation, shear stress or heat. Chemical and/or enzymic degradation are preferred, with enzymic degradation being most preferable, e.g. using an amylase and/or glucosyl transferase, suitably one or more enzyme selected from the group comprising: cyclomaltodextrinase (Cdase, EC 3.2.1.54), Neopullulanase (Npase, EC 3.2.1.135) and maltogenicamylase (Mase, EC 3.2.1.133). Alternatively or additionally removal of the cyclodextrin(s) and/or derivative(s) thereof can be performed by precipitation, dilution, buffer exchange through dialysis, diafiltration, filtration and/or a chromatographic method (such as gel permeation, size exclusion chromatography, ion exchange chromatography, or affinity chromatography).

Also encompassed within protein folding methods of the invention are methods in which one or more chitin(s), chitin derivative(s), chitosan(s), and/or chitosan derivative(s), cycloamyloses(s) and/or cycloamyloase derivatives(s) is included in the protein folding solution. The one or more chitin(s), chitin derivative(s), chitosan(s), chitosan derivative(s), cycloamyloses(s) and/or cycloamyloase derivatives(s) is/are preferably introduced into the protein folding solution during the folding process, e.g. after an initial incubation period. Protein folding
methods of the invention may involve a multi-step incubation, e.g. a two step incubation, in which the protein is initially incubated with one or more inulin and/or inulin derivative (e.g. for 30 minutes, 1, 2, 4, 6, 8 or 12 hours) and then one or more chitin(s), chitin derivative(s), chitosan(s), chitosan derivative(s), cycloamyloses(s) and/or cycloamyloase derivatives(s) can be introduced into the solution and the incubation continued. These methods may further comprise removal of the chitin(s), chitin derivative(s), chitosan(s) chitosan derivative(s), cycloamyloses(s) and/or cycloamyloase derivatives(s) from the solution; during and/or after protein folding.

Removal of one or more of the chitin(s), chitin derivative(s), chitosan(s) chitosan derivative(s) cycloamyloses(s) and/or cycloamyloase derivatives(s) can be carried out by degradation, e.g. by one or more of the following methods: chemical degradation, enzymic degradation, electromagnetic radiation, shear stress or heat, but is preferably performed by chemical and/or enzymic degradation. Enzymic degradation may involve the use of one or more enzyme selected from the group comprising: chitinase (EC 3.2.1.14), Lysozyme (EC 3.2.1.17), chitosanase (EC 3.2.1.132) and β-N-acetylhexosaminidase (EC 3.2.1.52). Alternatively or additionally, removal of the chitin(s), chitin derivative(s), chitosan(s) chitosan derivative(s) cycloamyloses(s) and/or cycloamyloase derivatives(s) can be achieved by precipitation, dilution, buffer exchange through dialysis, diafiltration, filtration and/or a chromatographic method (such as gel permeation, size exclusion chromatography, ion exchange chromatography, or affinity chromatography).

Method for folding protein which incorporate inulin(s) or derivatives thereof may be preceded by using a chaotrope, detergent and/or reducing agent to denature protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution of protein in non-native conformation (fully or partially denatured protein). Suitable chaotropes include one or more of guanidine hydrochloride and/or urea. The reducing agent can be one or more of dithiothreithiol, dithioerythritol, beta-mercaptoethanol and Tris (2-carboxyethyl)
phosphine hydrochloride (TCEP.HCl). The detergent employed can be one or more of an anionic detergent (e.g. lauroyl sarcosine, SDS), a cationic detergent (e.g. cetyl trimethylammonium bromide (CTAB)), a non-ionic detergent (e.g. Triton®-X 100, Tween 60 (Sorbitan), dodecylmaltoside and/or a zwitterionic detergent (e.g. CHAPS). Suitable conditions, concentrations and combinations of denaturants such as chaotropes, detergents and/or reducing agents for denaturing proteins are well known in the art.

After denaturation, the concentration of chaotrope, detergent and/or reducing agent can be reduced by dilution or buffer exchange through dialysis, diafiltration, filtration, and/or a chromatographic method or methods such as gel permeation, size exclusion, chromatography, ion exchange chromatography or affinity chromatography. The concentration of chaotrope, detergent and/or reducing agent can be reduced prior to, at the start of and/or during the method for folding protein. Ideally the concentration of chaotrope, detergent and/or reducing agent is reduced to a level that would permit the native conformation to exist in solution, e.g. typically below 2 M urea or below 1 M guanidine hydrochloride.

In protein folding methods of the invention in which an inulin or derivative thereof is employed as a or the folding control compound, one or more disulphide shuffling agent(s) can be included in the folding solution. Suitable disulphide shuffling agents for use in methods of the invention include reduced and oxidised glutathione, cysteine, cystine and disulphide isomerases preferably at a concentration in the range of from 10µM to 10mM. Disulphide isomerases are preferably used at a concentration of from 10µM to 10 mM.

The choice of inulin or derivative thereof for use in methods of the invention is limited by the solubility of the inulin or derivative in the folding reaction conditions. Suitably the inulin or inulin derivative has a degree of polymerisation of from about 3 to about 500, about 3 to about 250 or about 3 to about 100, preferably from about 3 to about 50, more preferably from about 10
to about 50, yet more preferably from about 15 to about 40, further preferably from 20 to 30, e.g. 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30. It is preferred that inulin derivatives are derivatised by one or more type(s) of non-polar hydrocarbyl group, e.g. those selected from the group comprising a linear alkyl derivative(s), branched alkyl derivative(s) or a mixture of linear alkyl derivative(s) and branched alkyl derivative(s).

A preferred inulin or inulin derivative for use in a method of the invention is a compound of formula (I):

\[ G(O-CO-NH-R^1)_a [F(O-CO-NH-R^2)_b]_n \]  \hspace{1cm} (I)

wherein:

- G is a terminal glucosyl unit in which one or more hydroxyl groups thereof may be substituted with a group or groups of formula (O-CO-NH-R^1);
- R^1 is a straight or branched chain saturated or unsaturated hydrocarbyl group having from 1 to 25 carbon atoms and, where there is more than one (O-CO-NH-R^1) group on the glucosyl unit, each R^1 group may be the same or different;
- a is an integer of from 0 to 4;
- F is a fructosyl unit in which one or more hydroxyl groups thereof may be substituted with a group or groups of formula (O-CO-NH-R^2);
- R^2 is a straight or branched chain saturated or unsaturated hydrocarbyl group having from 1 to 25 carbon atoms and, where there is more than one (O-CO-NH-R^2) group on the fructosyl unit, each R^2 group may be the same or different;
- b is an integer of from 0 to 3 and from 0 to 4 for the terminal fructosyl unit; and
- n is an integer of from 2 to 499 preferably of from 2 to 249, more preferably 2 to 99, yet more preferably 2 to 49, further preferably 9 to 49, yet further preferably 14 to 39, most preferably 19-24;
each unit of formula \( F(O-CO-NH-R^2)_b \) may be the same or different from any other unit of formula \( F(O-CO-NH-R^3)_b \); and

the average degree of substitution per glucosyl or fructosyl unit is from 0.02 to 3.0.

Each group \( R^1 \) and \( R^2 \) may be selected from alkyl, alkenyl and alkynyl groups having from 1 to 25, preferably 3 to 22, most preferably 3 to 18 carbon atoms. One or more of the groups \( R^1 \) and \( R^2 \) can be an alkyl group having from 1 to 25, preferably 3 to 22, most preferably 3 to 18 carbon atoms; suitably one or more of groups \( R^1 \) and \( R^2 \) is an alkenyl or alkynyl group having from 1 to 25, preferably 3 to 22, most preferably 3 to 18 carbon atoms. Each alkyl group \( R^1 \) and \( R^2 \) can be a linear alkyl group having from 1 to 25, preferably 3 to 22, most preferably 3 to 18 carbons or branched alkyl group having from 3 to 25-, preferably 3 to 22, most preferably 3 to 18 carbons.

The average degree of substitution per glucosyl or fructosyl unit is suitably from 0.02 to 3.0, preferably from 0.05 to 1.0, most preferably from 0.05 to 0.5. The compound of formula (I) can be a polydisperse linear or slightly branched inulin N-alkylurethane, e.g. selected from the group consisting of inulin N-n-octylcarbamates, inulin N-n-dodecylcarbamates and inulin N-n-octadecylcarbamates. A suitable inulin derivative of formula (I) is Inutec® SP1 (Orafti, Tienen, Belgium).

The present invention provides a method for removing a folding control compound from an aqueous solution comprising protein, said method comprising degradation of the folding control compound, wherein said folding control compound is not cyclodextrin and said aqueous solution comprising protein is not a food system. Also provided is a method for removing a folding control compound from an aqueous solution comprising protein, said method comprising degradation of the folding control compound, wherein said folding control compound is not cyclodextrin and said protein is not an egg yolk protein.
Further provided is method for removing an inulin or a derivative thereof from an aqueous solution comprising protein, said method comprising degradation of said inulin and/or derivative thereof. In such methods the aqueous solution comprises protein in one or more of a fully denatured form, a partially folded form; a native conformation, an aggregated form or an inclusion body. These methods are particularly useful for removal of folding control compounds for solutions during and after protein refolding.

The folding control compound can be a polymer and is preferably a sugar polymer as described herein, removal is preferably by enzymic degradation of the sugar polymer.

Additionally, the present invention provides a method for degrading polymer(s) capable of modifying protein structure comprising:

(a) contacting a protein with one or more of said polymer(s), and,

(b) degradation of the one or more polymer(s) to reduce or eliminate the structure-modifying properties of the polymer(s).

Also provided is a method for controlling degradation of polymer(s) capable of modifying protein structure comprising:

(a) contacting a protein with one or more polymer(s), and,

(b) controlled degradation of the one or more polymer(s) to reduce or eliminate the structure-modifying properties of the polymer(s).

Further provided is a method for modifying protein structure comprising:

(a) in solution, contacting a protein with one or more polymer(s) capable of modifying protein structure, and,

(b) degradation of the one or more polymer(s) to reduce or eliminate the structure-modifying properties of the polymer, and thereby modify protein structure.

Yet further provided is a method for controlling protein structure comprising:
(a) in solution, contacting a protein with one or more polymer(s) capable of modifying protein structure, and,

(b) controlled degradation of the one or more polymer(s) to reduce or eliminate the structure-modifying properties of the polymer, and thereby modify protein structure.

The invention further provides novel kits and consumables suitable for use in methods of the invention. A kit is provided for performing a method of the invention, optionally together with instructions for use of the kit. A kit according to the invention may comprise, in a container, one or more folding control compound(s), together with instructions for use of the kit in a method of the invention. Also provided is a kit which comprises one or more folding control compound(s), preferably in a first container, and one or more substance(s) capable of degrading the folding control compound(s), preferably in a second container, optionally together with instructions for use of the kit.

In a preferred embodiment, a kit may comprise one or more folding control compound(s) in a container and a support means containing a support to which is attached a substance or substances capable of degrading the one or more folding control compound(s), or a kit may comprise one or more folding control compound(s) in a container or containers, and a support to which is attached a substance or substances capable of degrading the one or more folding control compound(s).

A folding control compound used in a kit according to the invention is preferably a polymer, more preferably a sugar polymer, more preferably a cyclic or helical sugar polymer, yet more preferably a fructosan or a glucosan as described herein. The folding control compound can be one or more cyclodextrin(s) or derivative(s) thereof and/or one or more inulin(s) or derivative(s) thereof. The folding control compound(s) and/or the substance(s) capable of degrading the folding control compound(s) may be provided in (aqueous) solution or in dry form. Thus the cyclodextrin(s) and/or the glucosyltransferase and/or amylase
may be provided in aqueous solution, e.g. in an appropriate buffer, or in dry form.

Preferably, in a kit according to the invention the one or more folding control compound is one or more cyclodextrin(s) and/or derivative(s) thereof, in that instance the kit may comprise one or more substance(s) capable of degrading cyclodextrin(s) and/or derivative(s) thereof, preferably an enzyme or enzymes, most preferably glucosyltransferase and/or an amylase. Accordingly, a kit may comprise one or more cyclodextrin(s) and/or derivative(s) thereof in a container or containers, and a support means containing a support to which glycosyltransferase and/or amylase is attached. A kit may comprise one or more cyclodextrin(s) and/or derivative(s) thereof in a container or containers, and a support to which glucosyltransferase and/or amylase is attached.

In alternative preferred embodiments of a kit according to the invention, the one or more folding control compound is one or more inulin(s) and/or derivative(s) thereof. In that instance, the kit may comprise one or more substance(s) capable of degrading the one or more inulin(s) and/or derivative(s), preferably an enzyme or enzymes, more preferably an exo-inulinase and/or endo-inulinase. Thus a kit may comprise one or more inulin(s) and/or derivative(s) thereof in a container or containers and a support means containing a support to which is attached an exo-inulinase and/or endo-inulinase. A kit may comprise one or more inulin(s) and/or inulin derivative(s) in a container or containers, and a support to which is attached an exo-inulinase and/or endo-inulinase.

Kits containing one or more inulin(s) or derivative(s) thereof may further comprise a cyclodextrin and/or a derivative thereof, and/or a substance or substances capable of degrading a cyclodextrin and/or a derivative thereof, preferably an enzyme or enzymes, capable of degrading a cyclodextrin and/or a derivative thereof, most preferably glucosyltransferase and/or amylase.
A kit containing a cyclodextrin, inulin or derivative thereof may comprise one or more of a chitin, chitin derivative, a chitosan, a chitosan derivative, a cycloamylose, a cycloamylose derivative, a hydrophobic resin and/or a hydrophobic gel and or may comprise a substance or substances, capable of degrading a chitin, chitin derivative, chitosan, chitosan derivative, cycloamylose and/or cycloamyloase derivative, preferably an enzyme or enzymes, capable of degrading a chitin, chitin derivative, chitosan, chitosan derivative, cycloamylose and/or cycloamyloase derivative.

A support means suitable for use in a kit according to the invention can be a pipette tip, centrifuge tube or column. A support suitable for use in a kit according to the invention can be a resin, gel, beads, membrane, pipette tip, centrifuge tube, or column.

The invention also provides consumables suitable for use in methods of the invention. There is provided a support means containing a substance or substances capable of degrading a folding control compound or compounds, a support means containing a support to which is attached a substance or substances capable of degrading a folding control compound or compounds. The support means can be a pipette tip, centrifuge tube or column.

There is provided a support to which is attached a substance or substances capable of degrading a folding control compound or compounds, the support can be a resin, gel, beads, membrane, pipette tip, centrifuge tube, or column.

In a support means or support according to the invention the substance or substances contained within or attached thereto can be a substance or substances capable of degrading cyclodextrin(s) or derivative(s) thereof, such as an enzyme or enzymes, preferably glucosyl transferase and/or amylase.

In an alternative aspect of a support means or support according to the invention the substance or substances contained within or attached thereto are
capable of degrading inulin(s) or derivative(s) thereof, e.g. an enzyme or enzymes, preferably one or more exo-inulinase(s) and/or endo-inulinase(s).

Attachment, i.e. immobilisation, of a substance such as an the enzyme on the support means and/or support is preferably through covalent bonding, e.g. via the amine, thiol, carboxylic acid or aldehyde functional groups of the enzyme.

In methods, consumables and kits of the invention, degradation of the folding control compound can be achieved by contacting the folding control compound-protein solution with, or passing the folding control compound-protein solution through a pipette tip, tube or column containing a substance or substances capable of degrading the folding control compound. The substance(s) may be directly immobilised on the tip tube or column. Alternatively the pipette tip, tube or column may contain the substance(s) immobilised on a resin, gel, bead, membrane or other suitable supporting structure. Degradation of the folding control compound may also be achieved by contacting the folding control compound – protein solution with a resin, gel, bead, membrane, or other suitable supporting structure, to which is attached a substance or substances capable of degrading the folding control compound.

In the methods, consumables and kits of the invention, wherein a cyclodextrin is a, or the, folding control compound, degradation of the cyclodextrin can be by contacting the protein-cyclodextrin solution with, or passing the protein-cyclodextrin solution through a glucosyltransferase or amylase-containing pipette tip, tube or column. The glucosyltransferase or amylase enzyme may be directly immobilised on the tip tube or column. Alternatively the pipette tip, tube or column may contain glucosyltransferase and/or amylase immobilised on a resin, gel, bead or membrane or other suitable supporting structure. In a further alternative, degradation of the cyclodextrin can be achieved by contacting the cyclodextrin-protein solution with a resin, gel, bead, membrane or other suitable supporting structure to which is attached glucosyltransferase and/or amylase.
In methods, consumables and kits of the invention, degradation of the inulin(s) and/or inulin derivative(s) in the protein solution can be achieved by contacting the solution with, or passing the solution through a pipette tip, tube or column containing a substance or substances capable of degrading the inulin(s) and/or inulin derivative(s).

The substance(s) may be directly immobilised on the tip tube or column. Alternatively the pipette tip, tube or column may contain the substance(s) immobilised on a resin, gel, bead, membrane or other suitable supporting structure. Degradation of the inulin(s) and/or inulin derivative(s) in the protein solution may also be achieved by contacting the solution with a resin, gel, bead, membrane, or other suitable supporting structure, to which is attached a substance or substances capable of degrading the inulin(s) and/or inulin derivative(s).

In the methods, consumables and kits of the invention, degradation of the inulin(s) and/or inulin derivative(s) in the protein solution can be by contacting the solution with, or passing the solution through a endo-inulinase and/or exo-inulinase-containing pipette tip, tube or column. The enzyme(s) may be directly immobilised on the tip tube or column. Alternatively the pipette tip, tube or column may contain the enzyme(s) immobilised on a resin, gel, bead or membrane or other suitable supporting structure. In a further alternative, degradation of the inulin(s) and/or inulin derivative(s) in the protein solution can be achieved by contacting the solution with a resin, gel, bead, membrane or other suitable supporting structure to which is attached endo-inulinase(s) and/or exo-inulinase(s).
List of Figures

Figure 1 shows the aggregation kinetics for different rates of cyclodextrin (CD) breakdown catalysed by 2 µg/ml, 20 µg/ml or 200µg/ml diastase. Lysozyme was initially denatured with urea and then diluted 20x into a β-cyclodextrin containing phosphate buffer. The y-axis shows the absorbance of red light at 500nm, A500. This is a commonly used measure of aggregation. The x-axis shows time in minutes. The initial protein concentration was 5 mg/ml in 8M urea. This solution was then diluted or 20x into 67mM phosphate buffer at pH 6.5 containing 16.3mM β-cyclodextrin. The plot shows that β-cyclodextrin can be broken down by the action of the diastase enzyme to release the aggregation prone lysozyme refolding intermediates. The higher the diastase concentration, the faster the β-cyclodextrin breakdown and the greater the rate and overall level of lysozyme aggregation. The legend indicates the final diastase concentration. The plot shows that not all of the lysozyme refolded as the aggregation increases as the β-cyclodextrin is broken down, but the more gradual degradation of β-cyclodextrin at the lower diastase concentrations results in reduced turbidity (A500) indicating that less aggregation is taking place.

Figure 2 shows the aggregation occurring after lysozyme denatured in urea was diluted 20x into phosphate buffer. This experiment corresponds to that shown in figure 1, except that in this experiment the phosphate buffer does not contain β-cyclodextrin. The y-axis is the absorbance of red light at 500nm, the x-axis is time in minutes. The plot shows that in the absence of β-cyclodextrin, i.e. without the protective effect of β-cyclodextrin, the overall level of aggregation is increased. No significant change is observed in the aggregation between 50 and 200 minutes as there is no β-cyclodextrin remaining present upon which the diastase enzyme could act. Without β-cyclodextrin, the lysozyme is not maintained in a denatured form and all of the lysozyme is available for refolding or aggregation immediately after dilution.
Figure 3 shows dilution of denatured lysozyme into phosphate buffer performed in the same manner as the experiments shown in figure 1. Each experiment is performed with and without β-cyclodextrin in the phosphate buffer. The results have been normalised with the highest A500 value to provide a comparison of the aggregation kinetics. Each curve with β-cyclodextrin shows a rising A500 value as the lysozyme is released from the β-cyclodextrin to complete the refolding process or to aggregate. The control without β-cyclodextrin present generally show flatter A500 profiles as all the lysozyme is available for refolding and aggregation immediately after the initial dilution.

Figure 4(a) shows reduction in turbidity between the β-cyclodextrin containing samples and the non-β-cyclodextrin containing controls at the end of the experiments shown in figure 3. The plot shows an optimum between 0.01 and 1 ug/ml diastase indicating that the rate of β-cyclodextrin breakdown achieved at this enzyme concentration results in the minimum level of lysozyme aggregation.

Figure 4(b) shows the enzymatic activity of the lysozyme samples refolded with β-cyclodextrin. The plot shows an optimum around 1 ug/ml diastase indicating that the total enzyme activity is maximised at the rate of β-cyclodextrin release achieved by this enzyme concentration. The samples refolded without any β-cyclodextrin in the refolding buffer did not show any enzymatic activity.

Figure 5 shows the refolding yields for lysozyme (0.25 mg/ml final concentration) after 20 fold dilution into refolding buffer containing inulin and inulinase. The y-axis is % yield of native functional lysozyme enzyme after folding, the x-axis is final inulinase concentration. The pale grey bars indicate samples containing 0mg/ml inulin, the dark grey bars indicate samples that initially contained 10 mg/ml inulin. The samples were incubated for 24 hours at room temperature before the refolding measurements were taken. The presence of 10mg/ml inulin (Inutec N®25, Orafti, Belgium) enhanced the yield of
lysozyme for all inulnase concentrations except the very highest, 50 units/ml, at that concentration the yields in the presence and absence of inulin were approximately equal.

Examples

Example 1: Lysozyme refolding through controlled degradation of cyclodextrin by diastase (amylase)

Methodology
Aggregation was monitored by measuring the turbidity of a solution, that is, the amount of red light absorbed/scattered by the solution. The more protein aggregates, the higher the turbidity reading. For the experiments discussed below, the turbidity was monitored at 500 nm (A500).

Denatured protein was diluted as described below in 67mM phosphate buffer pH 6.5 containing a high concentration (16.3mM) of β-cyclodextrin (BCD), or in 67mM phosphate buffer pH 6.5 without BCD (control). The turbidity difference after dilution in the different buffers was compared. Maintenance of protein in a non-native, non-aggregated, conformation by BCD was detected as a reduction in the turbidity relative to controls. Following this step, a sample of the protein-BCD solution was subjected to enzymatic degradation of the BCD by diastase digestion. An increase in turbidity indicated that both aggregation and refolding were taking place on degradation of BCD, demonstrating that BCD had acted to inhibit protein aggregation and refolding. Finally an enzyme activity assay was performed to determine the yield of active protein.

Lysozyme was used a model protein in folding aggregation experiments. Lysozyme is a monomeric protein of 14.4 kDa. It contains four disulphide bonds. Lysozyme was used because it is a well known, easy to analyse, cheap and readily available protein.
Denatured protein solution
A denatured lysozyme stock of 20 mg/ml was made by dissolving 200 mg of native lysozyme in 10 ml denaturant buffer containing 8 M urea, 32 mM DTT, 0.1 M Tris, 1 mM EDTA at pH 8.1. This solution was left overnight at room temperature to allow the protein to denature fully. Next, the protein was diluted to a stock of 5 mg/ml denatured protein with denaturant buffer.

BCD solution
A BCD solution was made containing 16.3 mM BCD, 10 mM reduced glutathione (GSH) and 1 mM oxidised glutathione (GSSG) in 67 mM phosphate at pH 6.5.

Control solution
For control experiments (no BCD) a 10 mM GSH and 1 mM GSSG in 67 mM phosphate buffer at pH 6.5 was used.

Diastase solution
A diastase stock of 20 mg/ml was made by dissolving 200 mg of diastase in 10 ml of 67 mM phosphate buffer at pH 6.5. A log dilution series was prepared using the same buffer down to a final protein concentration of 20 x 10^-7 mg/ml.

Experiment 1
Denatured protein (12.6 µl at 5 mg/ml) was diluted 20 x in 239.4 µl BCD (16.3 mM) solution at pH 6.25, or control solution (no BCD) at pH 6.25. Following this, 28 µl of diastase solution was added resulting in a total reaction volume of 280 µl with a final lysozyme concentration of 0.23 mg/ml and when present, a final BCD concentration of 14.7 mM. This was repeated three times, for each diastase concentration, for both the BCD and control case. The final lysozyme concentration in the reaction mixtures was 0.23 mg/ml. The turbidity (A500nm) of the samples was measured at 25 minute intervals after dilution (Figure 1, Figure 2). All reactions were carried out at room temperature. The turbidity readings were averaged and normalised to the maximum turbidity reading (A0)
observed for the 'no-BCD' control samples for each diastase concentration (Figure 3).

The difference (delta) between the turbidity of the 'BCD' samples and the 'no-
BCD' controls corresponds to a reduction of protein aggregation, as shown in
figure 3, as the diastase concentration is reduced from 2000 µg/ml, the
difference between the CD and no CD curves at 200 minutes increases. The
delta turbidity value is the difference between the two curves at 200 minutes
expressed as a percentage of the no CD control.

Aggregation in the 'BCD' samples increased on digestion of BCD indicating that
the denatured protein had been protected from aggregation in a BCD-protein
complex. The rate at which BCD is digested determines the degree of
aggregation of protein. For fast degradation i.e. achieved with 2000 µg/ml
diastases, no major difference in turbidity was observed. For lower degradation
rates, i.e. achieved with 2 µg/ml diastase aggregation was significantly lower in
the amylase digested BCD samples when compared to the 'no-BCD' control
samples, showing that the pool of soluble native-like protein was increased by
BCD protection of denatured protein, followed by amylase digestion of the BCD.
An optimum degradation rate for BCD, was achieved using 0.02 µg/ml diastase,
indicating that controlling the rate of degradation of BCD controls protein
refolding and reduces the formation of aggregates, thereby maximising the
refolding yield.

Lysozyme activity was measured at room temperature by following the
decrease in absorbance at 490 nm of a cell suspension (0.60 mg/ml
*Micrococcus lysodeikticus*, 67 mM phosphate, pH 6.25). 20 µl of refolded
lysozyme was diluted with 260 µl of 0.60 mg/ml *Micrococcus lysodeikticus* in 67
mM phosphate buffer at pH 6.25. The absorbance was observed for 1.5 min. A
linear decrease in absorbance was observed. The concentration of refolded
lysozyme was determined by comparing the activity of the refolded lysozyme to
the activity of standard solutions of native lysozyme of different concentrations.
For the controls without BCD in the refolding buffer, no lysozyme activity was observed. For the BCD samples, active, refolded lysozyme was obtained. The rate of BCD degradation using diastase determines the yield of active lysozyme (Figure 4).

These results demonstrate:
(i) that β-cyclodextrin (BCD) can maintain denatured protein in a non-native conformation in an environment that would otherwise, in the absence of BCD, induce both refolding and irreversible aggregation.
(ii) that enzymes can be used to degrade cyclodextrin in protein-cyclodextrin complexes in solution.
(iii) that enzymes can be used to induce folding/refolding/aggregation of proteins from BCD/protein complexes.
(iv) that steps (ii) and (iii) can be carried out in a controlled manner whereby the rate of degradation affects both the refolding yield and degree of aggregation.

**Example 2: Refolding Lysozyme using inulin**

Lysozyme (Fluka 62971) was denatured overnight at 4°C in 100 mM Tris-HCl buffer at pH 8.1, containing 1 mM EDTA, 8 M urea and 32 mM DTT. The denatured protein (5.0 to 20.0 mg/ml) was diluted 20 times into a refolding buffer (67 mM Tris, 5 mM oxidized glutathione, pH 6.5). In half of the samples, the refolding buffer also contained 10 mg/ml inulin (Inutec N®25, Orafti, Belgium). After dilution of the lysozyme into the refolding buffer the enzyme inulinase (Fructozyme, 2000 units/ml, Novozymes) was added. The inulinase was diluted from stock to concentrations ranging from 0.5 – 500 units/ml and 10% by volume was added to each sample, in the well of a 96-well microplate.

The samples were then incubated for 24 hr at room temperature (approx. 25°C) to allow refolding. Lysozyme activity (protein folded to a native, active
configuration) was determined by measuring the initial linear rate of decrease in
turbidity (absorption at 492 nm) of a Micrococcus lysodeikticus (Sigma M-3770)
solution. The Micrococcus concentration was 0.75 mg/ml in 67 mM sodium
phosphate buffer at pH 6.25. The Micrococcus solution was mixed with the
solution containing the refolded lysozyme in the ratio 10:1 (e.g. 200 μl
Micrococcus solution, 20 μl refolded lysozyme solution). Standards of native
(non-refolded) lysozyme were used to generate a standard curve to calculate
the refolding yield.

Figure 5 shows the refolding yields for lysozyme refolded at a final protein
concentration of 0.25 mg/ml in sodium phosphate buffer. In half of the samples,
the refolding buffer contained 10 mg/ml inulin. The enzyme inulinase was
added to the refolding buffer at various concentrations to degrade the inulin
polymer. The presence of 10 mg/ml inulin enhanced the refolding yield of the
lysozyme compared with the control data where no inulin was present in the
refolding buffer. Inulin improved the refolding yield in all cases except when the
enzyme inulinase was present at very high concentration. The results for the
control case without inulin suggest that the lysozyme activity assay or the
lysozyme activity itself is inhibited in the presence of high concentrations of
inulinase. This may be due to a contaminant that is present in the concentrated
inulinase solution used to perform the experiments.

Example 3 Protein Folding Kit

Kit contents

Denaturation buffer: 100 mM Tris, 8 M urea, 32 mM DTT, 1mM EDTA, pH 8.1
(14 ml).
Protein protection/Refolding buffer: 67 mM Tris.Cl, 5 mM GSSG, pH 6.5, 10
mg/ml inulin (Inutec® N25, Orafti, Belgium.) (7 ml).
Refolding tubes: 3x 2.0 ml centrifuge tubes.

Protection removal resin: 3 x 400 μl sepharose resin with amine immobilised
inulinase, supplied as 50% slurry in 67 mM Tris.Cl, 5 mM GSSG, pH 6.5,
Insert of a spin column, Wash collection tube (component of spin column), product collection tube (component of spin column).

Instructions for use

1. Use the denaturation buffer to solubilise the protein or inclusion body pellet. The target protein concentration is 5 mg/ml of solubilised and denatured protein. Agitation (e.g. vortex mixing, shaking platform) will be required to dissolve the protein pellet. Incubate overnight at 4°C (or 3 hours at 37°C) to fully denature the protein and finally centrifuge to pellet any remaining insoluble material.

2. Add 475 µl of the protection/refolding buffer to one of the refolding tubes. If desired an alternative or proprietary refolding buffer can be substituted at this stage.

3. Add 25 µl of the solubilised and denatured protein and immediately vortex mix to begin the protein protection process and immediately vortex mix to begin the refolding process. Refolding should be performed overnight at room temperature.

4. Resuspend the resin in the spin column insert by vortex mixing, remove the top cap and remove the column tip. Place the insert into the wash collection tube.

5. Centrifuge the assembly for 1 min at 80 g to settle the resin and remove the excess liquid.

6. Remove the insert from the wash collection tube and add a cap to the column tip. Add the refolding protected protein from step 3, to the top of the insert and vortex mix to resuspend the resin enabling completion of the refolding process.

7. Incubate for up to 6 hours at room temperature.

8. Remove the cap from the column tip and place the insert into the product collection tube.

9. Centrifuge the assembly for a further 1 min at 80 g. The refolded protein product is now contained in the product collection tube.
CLAIMS:

1. A method for folding protein comprising:
(a) in aqueous solution, contacting denatured protein with one or more folding control compounds(s), and
(b) degradation of the folding control compound(s).

2. A method for controlling protein folding comprising:
(a) in aqueous solution, contacting denatured protein with one or more folding control compound(s), and
(b) controlled degradation of the folding control compound(s).

3. A method for folding protein comprising:
(a) providing an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more folding control compound(s) present at a concentration sufficient to maintain protein in denatured conformation, and
(c) degradation of the folding control compound(s), to allow the protein to fold.

4. A method for folding protein comprising:
(a) using a chaotrope, detergent, and/or reducing agent to denature protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more folding control compound(s) present at a concentration sufficient to maintain protein in denatured conformation,
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the folding control compound(s), to allow the protein to fold.

5. A method according to claim 4 wherein the chaotrope is one or more of guanidine hydrochloride and/or urea.
6. A method according to claim 4 or claim 5, wherein the reducing agent is one or more of dithiothreithiol, dithioerythritol, beta-mercaptopethanol or Tris (2-carboxyethyl) Phosphine Hydrochloride (TCEP.HCl).

7. A method according to claim 4, 5, or 6, wherein the detergent is one or more of an anionic detergent, a cationic detergent, a non-ionic detergent and/or a zwitterionic detergent, Sodium dodecyl sulfate, Octoxynol-9 (Triton®-X 100), or a Sorbitan.

8. A method according to any one of claims 4 to 7, wherein the concentration of chaotrope, detergent and/or reducing agent is reduced by dilution or buffer exchange through dialysis, diafiltration, filtration, and/or a chromatographic method, gel permeation, size exclusion, chromatography, ion exchange chromatography and/or affinity chromatography.

9. A method according to any preceding claim, wherein the folding control compound is a polymer.

10. A method according to any preceding claim, wherein the folding control compound is a sugar polymer or a derivative thereof.

11. A method according to any preceding claim, wherein the folding control compound is a linear or non-linear amphipathic sugar polymer or a derivative thereof.

12. A method according to any preceding claim, wherein the sugar polymer comprises one or more sugar selected from the group consisting of: glucose, fructose, mannose, and/or galactose.
13. A method according to any preceding claim, wherein the sugar polymer is a dextran, cellulose, amylose, starch, pullulan, mannan, chitin, chitosan, inulin, levan, xylan, cyclodextrin, cycloamylose or a derivative thereof.

14. A method according to any one of claims 10 to 13, wherein the sugar polymer is derivatised with one or more substituents selected from the group consisting of: a linear alkyl chain, branched alkyl chain, double or triple bonded hydrocarbon, aromatic group, fatty acid and a polyol.

15. A method according to claim 10, wherein the folding control compound is a cyclic sugar polymer.

16. A method according to claim 15, wherein the folding control compound is a glucosan.

17. A method according to claim 16, wherein the folding control compound is a cyclodextrin or derivative thereof.

18. A method according to claim 16 or claim 17, wherein the folding control compound is a α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin, or a derivative thereof.

19. A method according to claim 17 or claim 18, wherein the initial concentration of folding control compound is in the range of from about 100 to about 10,000 times the molar concentration of protein.

20. A method according to claim 10, wherein the folding control compound is a helical sugar polymer.

21. A method according to claim 20, wherein the folding control compound is a fructosan.
22. A method according to claim 21, wherein the folding control compound is an inulin or a derivative thereof.

23. A method according to claim 22, wherein the folding control compound is an inulin or a derivative thereof with a degree of polymerisation of from about 3 to about 500, about 3 to about 250, about 3 to about 100, about 3 to about 50, about 10 to about 50, about 15 to about 40, or from about 20 to about 30.

24. A method according to claim 22 or claim 23, wherein the folding control compound is an inulin derivative substituted with one or more type(s) of non-polar hydrocarbaryl group.

25. A method according to claim 24, wherein the one or more type(s) of non-polar hydrocarbaryl group is selected from the group comprising: linear alkyl derivative(s) and branched alkyl derivative(s).

26. A method according to any one of claims 22 to 25, wherein the initial concentration of folding control compound is in the range of from about 1 to about 1000 times the molar concentration of protein.

27. A method according to any one of claims 22 to 26, wherein the initial concentration of folding control compound is in the range of from about 1 to about 100 times the molar concentration of protein.

28. A method according to any preceding claim, wherein degradation of the folding control compound(s) is by one or more of the following methods: enzymic digestion, chemical degradation, electromagnetic radiation, shear stress and/or heat.

29. A method according to any one of claims 10 to 28, wherein degradation is by enzymic digestion.
30. A method according to any one of claims 17 to 19, wherein degradation is by glucosyltransferase and/or amylase digestion.

31. A method according to any one of claims 22 to 27, wherein degradation is by exo-inulinase and/or endo-inulinase digestion.

32. A method according to any preceding claim wherein a disulphide shuffling agent is present during degradation of the folding control compound.

33. A method according to claim 25, wherein the disulphide shuffling agent is selected from the group consisting of: a reduced or oxidised glutathione, cysteine, cystine and a disulphide isomerase.

34. A method for folding protein comprising:
   (a) in aqueous solution, contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, and
   (b) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

35. A method for controlling protein folding comprising:
   (a) in aqueous solution, contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, and
   (b) controlled degradation of the cyclodextrin(s) and/or derivative(s) thereof.

36. A method for folding protein comprising:
   (a) providing an aqueous solution comprising denatured protein,
   (b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present in solution at a concentration sufficient to maintain protein in denatured conformation, and
   (c) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.
37. A method for refolding protein comprising:
(a) using a chaotrope, detergent, and/or reducing agent to denature protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution comprising denatured protein,
(b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present at a concentration sufficient to maintain protein in denatured conformation,
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

38. A method for refolding protein comprising:
(a) providing an aqueous solution comprising denatured protein using a chaotrope, detergent and/or reducing agent to denature protein and/or dissolve aggregated protein and/or inclusion bodies,
(b) providing to the aqueous protein solution of (a) one or more cyclodextrin(s) and/or derivative(s) thereof, at a concentration sufficient to maintain the protein essentially in non-native conformation, preferably by buffer exchange to a solution containing one or more cyclodextrin(s) and/or derivative(s) thereof.
(c) reducing the concentration of the chaotrope, detergent and/or reducing agent, and
(d) degradation of the one or more cyclodextrin(s) and/or derivative(s) thereof, to allow the protein to refold.

39. A method according to claim 37 or 38, wherein the chaotrope is one or more of guanidine hydrochloride and/or urea.

40. A method according to any one of claims 37 to 39, wherein the reducing agent is one or more of dithiothreithiol, dithioerythritol, beta-mercaptoethanol or Tris (2-carboxyethyl) phosphine hydrochloride (TCEP.HCl).
41. A method according to any one of claims 37 to 40, wherein the detergent is one or more of SDS, Octoxynol-9 (Triton®-X 100), or a Sorbitan.

42. A method according to any one of claims 37 to 41 wherein the concentration of chaotrope, detergent and/or reducing agent is reduced by dilution, or buffer exchange through dialysis, diafiltration, filtration, and/or a chromatographic method, gel permeation, size exclusion, chromatography, ion exchange chromatography and/or affinity chromatography.

43. A method for controlling protein aggregation during protein folding comprising:
   (a) providing an aqueous solution comprising denatured protein,
   (b) contacting denatured protein with one or more cyclodextrin(s) and/or derivative(s) thereof, present in aqueous solution at a concentration sufficient to maintain the protein in non-aggregated form, and
   (c) degradation of said one or more cyclodextrin(s) and/or derivative(s) thereof, in a controlled manner to induce protein folding.

44. A method according to any one of claims 34 to 43, wherein the initial concentration of cyclodextrin(s) and/or derivative(s) thereof, is in the range of from about 1 to about 200 mg/ml.

45. A method according to any one of claims 34 to 44 wherein the initial concentration of cyclodextrin(s) and/or derivative(s) thereof is in the range of from about 1 to about 100 mg/ml.

46. A method according to any one of claims 34 to 45, wherein the initial concentration of cyclodextrin(s) and/or derivative(s) thereof is in the range of from about 10 to about 50 mg/ml.
47. A method according to claim 46, wherein the initial concentration of cyclodextrin(s) and/or derivative(s) thereof is in the range of from about 10 to about 30 mg/ml.

5 48. A method according to claim 47, wherein the initial concentration of cyclodextrin(s) and/or derivative(s) thereof is about 20 mg/ml.

49. A method according to any one of claims 34 to 48, wherein degradation of the cyclodextrin(s) and/or derivative(s) thereof is by one or more of the following methods: enzymic digestion, chemical degradation, electromagnetic radiation, shear stress and/or heat.

50. A method according to any one of claims 34 to 49, wherein degradation of the cyclodextrin(s) and/or derivative(s) thereof is by enzymic digestion and/or chemical degradation.

51. A method according to any one of claims 34 to 50, wherein degradation is by glucosyltransferase and/or amylase digestion.

52. A method according to any one of claims 34 to 51, wherein a disulphide shuffling agent is present during degradation of the cyclodextrin(s) or derivative(s) thereof.

53. A method according to claim 52, wherein the disulphide shuffling agent is selected from the group consisting of: a reduced or oxidised glutathione, cysteine, cystine and a disulphide isomerase.

54. A method for folding protein comprising:
(a) providing an aqueous solution comprising protein in non-native conformation and an inulin or inulin derivative or a mixture of inulin(s) and/or inulin derivative(s), and,
(b) degradation of the inulin(s) and/or inulin derivative(s), to permit folding of the protein.

55. A method for controlling protein folding comprising:
(a) providing an aqueous solution comprising protein in non-native conformation and an inulin or inulin derivative or a mixture of inulin(s) and/or inulin derivative(s), and,
(b) controlled degradation of the inulin(s) and/or inulin derivative(s), to permit folding of the protein.

56. A method according to claim 54 or 55, wherein the inulin or inulin derivative or mixture of inulin(s) and/or inulin derivative(s) is present initially at a low to medium concentration.

57. A method according to claim 56, wherein the inulin or inulin derivative or mixture of inulin(s) and/or inulin derivative(s) is present initially at a low to medium concentration in the range of from about 0.002 mg/ml to about 20 mg/ml.

58. A method according to claim 56, wherein the inulin or inulin derivative or mixture of inulin(s) and/or inulin derivative(s) is present initially at a low to medium concentration in the range of from about 0.01 mg/ml to about 5mg/ml.

59. A method according to any one of claims 54 to 58, wherein degradation of the inulin(s), and or inulin derivative(s) is by one or more of the following methods: enzymic digestion, chemical degradation, electromagnetic radiation, shear stress and/or heat.

60. A method according to any one of claims 54 to 59 wherein degradation of the inulin(s) and/or derivatives is by enzymic and/or chemical digestion.
61. A method according to any one of claims 54 to 60, wherein degradation of the inulin(s) and/or derivative(s) is by enzymic digestion.

62. A method according to any one of claims 54 to 61, wherein degradation of the inulin(s) and/or derivative(s) is by enzymic digestion using one or more exo-inulinase(s) and/or endo-inulinase(s).

63. A method according to claim 62, wherein the exo-inulinase(s) is Fructan β-fructosidase E.C.3.2.1.80 and/or the endo-inulinase(s) is inulinase E.C.3.2.1.7.

64. A method according to any one of claims 54 to 63, further comprising:
(c) removal of the inulin(s) and/or derivative(s) thereof by dilution, buffer exchange through dialysis, diafiltration, filtration, precipitation and/or a chromatographic method, gel permeation, size exclusion chromatography, ion exchange chromatography, and/or affinity chromatography.

65. A method according to any one of claims 54 to 64, wherein one or more of a chitin, chitosan, cyclodextrin, cycloamylose or a derivative thereof, a hydrophobic resin and/or a hydrophobic gel is included in the solution.

66. A method according to claim 65, further comprising:
(c) removal of one or more of the chitin, chitosan, cyclodextrin, cycloamylose, or a derivative thereof, a hydrophobic resin and/or a hydrophobic gel from the solution.

67. A method according to any one of claims 54 to 66, wherein a cyclodextrin, a cyclodextrin derivative or a mixture of cyclodextrin(s) and/or cyclodextrin derivative(s) is included in the solution.

68. A method according to claim 67, further comprising:
(c) removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) from the solution.
69. A method according to claim 66 or claim 68, wherein the cyclodextrin(s) is/are removed from the solution during and/or after protein folding.

70. A method according to claim 67 or claim 69, wherein removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) is by degradation.

71. A method according to claim 66 or any one of claims 68 to 70, wherein removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) is by chemical and/or enzymic degradation.

72. A method according to claim 66 or any one of claims 68 to 71, wherein removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) is by enzymic degradation.

73. A method according to claim 66 or any one of claims 68 to 72, wherein removal of the cyclodextrin(s) and/or cyclodextrin derivative(s) is by amylase and/or glucosyl transferase digestion.

74. A method according to any one of claims 70 to 73 wherein enzymic degradation is performed using one or more enzyme selected from the group comprising: cyclomaltooligosaccharidase (Cdos, EC 3.2.1.54), Neopullulanase (Npase, EC 3.2.1.135), maltogenicamylase (Mase, EC 3.2.1.133) Cyclodextrin glycosyltransferase(CGTase, EC 2.4.1.19).

75. A method according to any one of claims 66 to 74, wherein removal of the cyclodextrin(s) and/or derivative(s) thereof is by precipitation, dilution, buffer exchange through dialysis, diafiltration, filtration and/or a chromatographic method.
76. A method according to any one of claims 54 to 75 wherein one or more chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof is included in the solution.

77. A method according to claim 76, further comprising removal of the chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof from the solution.

78. A method according to claim 77, wherein one or more of the chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof is/are removed from the solution during and/or after protein folding.

79. A method according to claim 77 or claim 78, wherein removal of one or more of the chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof is by degradation.

80. A method according to any one of claims 76 to 78, wherein removal of one or more of the chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof is by chemical and/or enzymic degradation.

81. A method according to claim 80, wherein enzymic degradation is performed using one or more enzyme selected from the group comprising: chitinase (EC 3.2.1.14), Lysozyme (EC 3.2.1.17), chitosanase (EC 3.2.1.132) and β-N-acetylmuramidase (EC 3.2.1.52).

82. A method according to any one of claims 77 to 78, wherein removal of the chitin(s), chitosan(s), cycloamylose(s) or derivative(s) thereof is by precipitation, dilution, buffer exchange through dialysis, diafiltration, filtration and/or a chromatographic method.

83. A method according to any one of claims 54 to 82, wherein said method is preceded by using a chaotrope, detergent and/or reducing agent to denature
protein and/or dissolve aggregated proteins and/or inclusion bodies to provide an aqueous solution of protein in non-native conformation.

84. A method according to claim 83, wherein the chaotrope is one or more of guanidine hydrochloride and/or urea.

85. A method according to claim 83 or claim 84, wherein the reducing agent is one or more of dithiothreithiol, dithioerythritol, beta-mercaptoethanol and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP.HCl).

86. A method according to any one of claims 83 to 85, wherein the detergent is one or more of an anionic detergent, a cationic detergent, a non-ionic detergent and/or a zwitterionic detergent.

87. A method according to any one of claims 83 to 86, wherein the concentration of chaotrope, detergent and/or reducing agent is reduced by dilution or buffer exchange through dialysis, diafiltration, filtration, and/or a chromatographic method (such as gel permeation, size exclusion, chromatography, ion exchange chromatography and/or affinity chromatography).

88. A method according to claim 87, wherein the concentration of chaotrope, detergent and/or reducing agent is reduced prior to, at the start of, or during the method for folding protein.

89. A method according to any preceding claim wherein a disulphide shuffling agent is included in the folding solution.

90. A method according to claim 89, wherein the disulphide shuffling agent is selected from the group consisting of: a reduced or oxidised glutathione, cysteine, cystine and a disulphide isomerase.
91. A method according to any one of claims 54 to 90, wherein the or an inulin or inulin derivative has a degree of polymerisation in the range of from about 3 to about 500.

92. A method according to any one of claims 54 to 91, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 3 to about 250.

93. A method according to any one of claims 54 to 92, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 3 to about 100.

94. A method according to any one of claims 54 to 93, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 3 to about 50.

95. A method according to any one of claims 54 to 94, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 10 to about 50.

96. A method according to any one of claims 54 to 95, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 15 to about 40.

97. A method according to any one of claims 54 to 96, wherein the or a inulin or inulin derivative has a degree of polymerisation in the range of from about 20 to about 30.

98. A method according to any one of claims 54 to 97, wherein the or a inulin is Inutec®N25.
99. A method according to any one of claims 54 to 97, wherein the inulin derivative is substituted with one or more type(s) of non-polar hydrocarbyl group.

100. A method according to claim 99, wherein the inulin derivative is substituted with one or more type(s) of non-polar hydrocarbyl group selected from the group comprising a linear alkyl derivative(s), branched alkyl derivative(s) or a mixture of linear alkyl derivative(s) and branched alkyl derivative(s).

101. A method according to claim 99, wherein the or a inulin or inulin derivative is a compound of formula (I):

\[ G(O-CO-NH-R^1)_a - [F(O-CO-NH-R^2)_b]_n \]  \((I)\)

wherein:

- \( G \) is a terminal glucosyl unit in which one or more hydroxyl groups thereof may be substituted with a group or groups of formula \((O-CO-NH-R^1)\);
- \( R^1 \) is a straight or branched chain saturated or unsaturated hydrocarbyl group having from 1 to 25 carbon atoms and, where there is more than one \((O-CO-NH-R^1)\) group on the glucosyl unit, each \( R^1 \) group may be the same or different;
- \( a \) is an integer of from 0 to 4;
- \( F \) is a fructosyl unit in which one or more hydroxyl groups thereof may be substituted with a group or groups of formula \((O-CO-NH-R^2)\);
- \( R^2 \) is a straight or branched chain saturated or unsaturated hydrocarbyl group having from 1 to 25 carbon atoms and, where there is more than one \((O-CO-NH-R^2)\) group on the fructosyl unit, each \( R^2 \) group may be the same or different;
- \( b \) is an integer of from 0 to 3 and from 0 to 4 for the terminal fructosyl unit;
n is an integer of from 2 to 499 preferably of from 2 to 249, 2 to 99, 2 to 49, 9 to 49, 14 to 39 or 19 to 24,
each unit of formula $F(O-CO-NH-R^2)_n$ may be the same or different from any
other unit of formula $F(O-CO-NH-R^2)_n$; and
the average degree of substitution per glucosyl or fructosyl unit is from
0.02 to 3.0.

102. A method according to claim 101, wherein each group $R^1$ and $R^2$ is
selected from alkyl, alkenyl and alkynyl groups having from 1 to 25, preferably 3
to 22, most preferably 3 to 18 carbon atoms.

103. A method according to claim 101 or claim 102, wherein one or more of
the groups $R^1$ and $R^2$ is an alkyl group having from 1 to 25, preferably 3 to 22,
most preferably 3 to 18 carbon atoms.

104. A method according to any one of claims 101 to 103, wherein one or
more of groups $R^1$ and $R^2$ is an alkenyl or alkynyl group having from 1 to 25,
preferably 3 to 22, most preferably 3 to 18 carbon atoms.

105. A method according to claim 104, wherein each alkyl group $R^1$ and $R^2$ is
a linear alkyl group having from 1 to 25, from 3 to 22, or from 3 to 18 carbons,
or a branched alkyl group having from 3 to 25, from 3 to 22, or from 3 to 18
carbons.

106. A method according to any one of claims 101 to 105, wherein the
average degree of substitution per glucosyl or fructosyl unit is from 0.02 to 3.0,
preferably from 0.05 to 1.0, most preferably from 0.05 to 0.5.

107. A method according to any one of claims 101 to 106, wherein the
compound of formula (I) is a polydisperse linear or slightly branched inulin N-
alkylurethane.
108. A method according to any one of claims 101 to 107, wherein the compound of formula (I) is selected from the group consisting of inulin N-n-octyl-carbamates, inulin N-n-dodecylcarbamates and inulin N-n-octadecylcarbamates.

109. A method according to any one of claims 101 to 108, the inulin derivative is Inutech® SP1.

110. A method for removing a folding control compound from an aqueous solution comprising protein, said method comprising degradation of the folding control compound, wherein said folding control compound is not cyclodextrin and aqueous solution comprising protein is not a food system.

111. A method according to claim 110, wherein said folding control compound is not cyclodextrin and said protein is not an egg yolk protein.

112. A method for removing an inulin or a derivative thereof from an aqueous solution comprising protein, said method comprising degradation of said inulin and/or derivative thereof.

113. A method according to any one of claims 110 to 112, wherein the aqueous solution comprises protein in one or more of a fully denatured form, a partially folded form; a native conformation, an aggregated form or an inclusion body.

114. A kit for performing a method according to any one of the preceding claims, optionally together with instructions for use of the kit.

115. A kit comprising one or more folding control compound(s) and one or more substance(s) capable of degrading the folding control compound(s), optionally together with instructions for use of the kit.
116. A kit according to claim 115, comprising one or more folding control compound(s) in a first container and one or more substance(s) capable of degrading the folding control compound(s) in a second container, optionally together with instructions for use of the kit.

117. A kit according to claim 115, comprising one or more folding control compound(s) in a container and a support means containing a support to which is attached a substance or substances capable of degrading the one or more folding control compound(s).

118. A kit according to claim 115, comprising one or more folding control compound(s) in a container or containers, and a support to which is attached a substance or substances capable of degrading the one or more folding control compound(s).

119. A kit according to any one of claims 115 to 118, wherein the one or more folding control compound is one or more cyclodextrin(s) and/or derivative(s) thereof.

120. A kit according to claim 119, wherein the one or more substance(s) capable of degrading cyclodextrin(s) and/or derivative(s) thereof is an enzyme or enzymes.

121. A kit according to claim 120, wherein the one or more substance(s) capable of degrading cyclodextrin(s) and/or derivative(s) thereof is a glucosyltransferase and/or an amylase.

122. A kit according to claim 121, comprising one or more cyclodextrin(s) and/or derivative(s) thereof in a container or containers, and a support means containing a support to which glycosyltransferase and/or amylase is attached.
123. A kit according to claim 121 or claim 122, comprising one or more cyclohexalin(s) and/or derivative(s) thereof in a container or containers, and a support to which glucosyltransferase and/or amylase is attached.

124. A kit according to any one of claims 115 to 118, wherein the one or more folding control compound is one or more inulin(s) and/or derivative(s) thereof.

125. A kit according to claim 124, wherein the one or more substance(s) capable of degrading the one or more inulin(s) and/or derivative(s) thereof is an enzyme or enzymes.

126. A kit according to claim 125, wherein the one or more substance(s) capable of degrading the one or more inulin(s) and/or derivative(s) thereof is an exo-inulinase and/or endo-inulinase.

127. A kit according to claim 124, comprising one or more inulin(s) and/or derivative(s) thereof in a container or containers and a support means containing a support to which is attached an exo-inulinase and/or endo-inulinase.

128. A kit according to claim 124, comprising one or more inulin(s) and/or inulin derivative(s) in a container or containers, and a support to which is attached an exo-inulinase and/or endo-inulinase.

129. A kit according to any one of claims 124 to 128, comprising a cyclohexalin and/or a derivative thereof.

130. A kit according to any one of claims 124 to 129, comprising a substance or substances capable of degrading a cyclohexalin and/or a derivative thereof.

131. A kit according to any one of claims 124 to 130, comprising an enzyme or enzymes, capable of degrading a cyclohexalin and/or a derivative thereof.
132. A kit according to claim 124 to 131, comprising glucosyltransferase and/or amylase.

133. A kit according to any one of claims 119 to 132, comprising one or more of a chitin, chitin derivative, chitosan, chitosan derivative, cycloamylose, cycloamylose derivative, hydrophobic resin and/or hydrophobic gel.

134. A kit according to any one of claims 119 to 133, comprising a substance or substances, capable of degrading a chitin, chitin derivative, chitosan, chitosan derivative, cycloamylose and/or cycloamylose derivative.

135. A kit according to any one of claims 119 to 134, comprising an enzyme or enzymes, capable of degrading a chitin, chitin derivative, chitosan, chitosan derivative, cycloamylose and/or cycloamylose derivative.

136. A kit according to any one of claims 114 to 135, comprising a support means which is a pipette tip, centrifuge tube or column.

137. A kit according to any one of claims 114 to 136, comprising a support which is a resin, gel, beads, membrane, pipette tip, centrifuge tube, or column.

138. A support means containing a substance or substances capable of degrading a folding control compound or compounds.

139. A support means containing a support to which is attached a substance or substances capable of degrading a folding control compound or compounds.

140. A support means according to claim 138 or claim 139, which is a pipette tip, centrifuge tube or column.
141. A support to which is attached a substance or substances capable of degrading a folding control compound or compounds.

142. A support according to claim 141, which is a resin, gel, beads, membrane, pipette tip, centrifuge tube, or column.

143. A support means according to any one of claims 138 to 140 or a support according to claims 141 or 142, wherein the substance or substances contained within or attached thereto are capable of degrading cyclodextrin(s) or derivative(s) thereof.

144. A support means or support according to claim 143, wherein the substance or substances capable of degrading cyclodextrin(s) or derivative(s) thereof is and enzyme or enzymes.

145. A support means or support according to claim 144, wherein the enzyme(s) is glucosyl transferase and/or amylase.

146. A support means according to any one of claims 138 to 140 or a support according to claims 141 or 142, wherein the substance or substances contained within or attached thereto are capable of degrading inulin(s) or derivative(s) thereof.

147. A support means or support according to claim 146, wherein the substance or substances capable of degrading inulin(s) or derivative(s) thereof is an enzyme or enzymes.

148. A support means or support according to claim 147, wherein the enzyme(s) is one or more exo-inulinase(s) and/or endo-inulinase(s).
Figure 1
Figure 3

(c)

[Graph showing enzyme activity over time for 20 ug/ml Diastase with CD and NO CD conditions.]

(d)

[Graph showing enzyme activity over time for 2 ug/ml Diastase with CD and NO CD conditions.]
Figure 3

(e)

0.2 ug/ml Diastase

(f)

0.02 ug/ml Diastase
Figure 4

4(a)

![Graph showing Δ Turbidity (%) vs ug/ml Diastase]

4(b)

![Graph showing Yield (%) vs ug/ml Diastase]