METHOD FOR CATALYSING A FUSION POLYPEPTIDE UNDERGOING A CLEAVAGE REACTION

TECHNICAL FIELD

This present invention generally relates to a method of increasing the reaction rate of a fusion polypeptide undergoing a cleavage reaction, such as a method to accelerate cleavage of fusion polypeptides for increasing the production rate of recombinant polypeptides.

BACKGROUND

Recombinant polypeptides are usually expressed as fusion polypeptides containing a fusion partner linked to the target polypeptide. The fusion partner is commonly employed to enhance stability, increase solubility and facilitate purification of the target polypeptide in applications as diverse as biochemical purification, immunodetection, protein therapies, vaccine development, functional genomics, analysis of protein trafficking, and analyses of protein–protein or protein–nucleic acid interactions.

In general, such fusion polypeptides must be cleaved to release the target polypeptide. This is particularly important for the production of pharmaceutical proteins where authenticity of the protein sequence is essential since presence of additional amino acid residues may affect structural, physical and functional properties of the proteins. The usefulness of such a cleavage procedure depends on the efficiency and precision of cleavage and its cost per unit activity.

Cleavage of a peptide bond in a fusion polypeptide can be effected chemically or enzymatically. Chemical cleavage often requires elevated temperatures and toxic compounds, which denature the target polypeptide and complicate downstream purification. Slow reactivity and incomplete cleavage are also problems frequently encountered in such chemical cleavage processes. The more commonly used chemical cleavage reagents include hydroxylamine, N-chlorosuccinimide (NCS), cyanogen bromide (CNBr), or a combination of these. Hydroxylamine cleaves at Asn-Gly, NCS at Trp, and CNBr at the C-terminal side of the Met residue.

Enzymatic cleavage, on the other hand, is a milder procedure that in general does not denature the protein. Several highly specific restriction proteases that have been used for this purpose include Factor Xa, enterokinase, thrombin, TEV protease and signal peptidases. Of these, Factor Xa and enterokinase are two of the more widely used cleavage

systems, with both enzymes and expression vectors commercially available from a variety of suppliers such as Amersham-Biosciences, Novagen, SIGMA, Roche, Invitrogen – Life Technologies and New England Biolabs.

Factor Xa specifically recognizes the four amino acid sequence of Ile-Glu[or Asp]-Gly-Arg↓ while enterokinase recognizes the five amino acid sequence of Asp-Asp-Asp-Asp-Lys↓. Thrombin preferentially cleaves at Leu-Val-Pro-Arg↓Gly-Ser. However, release of the target polypeptide component from a fusion polypeptide can be affected by various factors, for example: a) the adjacent amino acid residue/sequence at the cleavage site; b) the size, nature and conformation of the two fused components; c) the accessibility of the cleavage site; d) reaction conditions such as pH, temperature, enzyme-to-target polypeptide ratio and incubation period; and e) other components present in the cleavage reactant solution such as salts (eg. NaCl), surfactants (eg. Triton X-100), reducing agents (eg. dithiothreitol), denaturants (eg. SDS) and protease inhibitors (eg. PMSF).

In the case of enterokinase, for example, the enzyme will not cleave if the residue adjacent to its cleavage site is proline. The enzyme is usually active within a pH range of 4.5-9.5 and a temperature range of between 4-45°C. Moreover, complete cleavage of a fusion polypeptide containing an enterokinase recognition sequence in the linker moiety typically proceeds extremely slowly (24 hours or longer) or not at all. Such slow reactivity may also be encountered in cleavage reactions involving other restriction proteases or classes of restriction proteases, or a combination of these enzymes. Use of higher enzyme quantities to increase rate of reaction is not only costly but may also result in unwanted proteolysis at secondary cleavage sites.

There is a need to provide a method to accelerate cleavage of fusion polypeptides that overcomes or at least ameliorates one or more of the disadvantages described above.

SUMMARY

According to a first aspect, there is provided a method of increasing the reaction rate of a fusion polypeptide undergoing a cleavage reaction, the method comprising the step of:

(a) providing an oxidant to a reactant solution of the fusion polypeptide as it undergoes the cleavage reaction to increase the rate of said reaction.

Advantageously, it has surprisingly been found by the inventors that with the addition of an oxidant, such as hydrogen peroxide, to a reactant solution comprising a fusion polypeptide undergoing a cleavage reaction, will increase the reaction rate. Advantageously, the increased rate of the cleavage reaction results in faster release of a target polypeptide of said fusion polypeptide.

In one embodiment, there is provided a method of increasing the reaction rate of a fusion protein undergoing a cleavage reaction, the method comprising the step of:

(a) providing hydrogen peroxide to a reactant solution of the fusion protein as it undergoes the cleavage reaction, wherein the fusion protein comprises at least one linker moiety connecting a polypeptide or peptide pair, said linker moiety having a cleavage recognition site for enterokinase.

In one embodiment, the fusion polypeptide comprises a number of amino acid residues selected from the group consisting of about 120 to about 190, about 160 to about 320, about 200 to about 400, about 350 to about 500, about 480 to about 600, and about 550 to about 650.

In one embodiment, the target polypeptide comprises a number of amino acid residues selected from the group consisting of about 3 to about 100, about 50 to about 170, about 90 to about 250, about 140 to about 300, about 180 to about 350, and about 220 to about 400.

In one embodiment, step (a) comprises the step of: (a1) providing the fusion polypeptide having at least one sulphur-containing amino acid residue.

In one embodiment, step (a) comprises the step of: (a2) providing the fusion polypeptide having at least one thiol-containing amino acid residue. The thiol-containing amino acid residue may be cysteine or a derivative thereof. The derivative may be D-cysteine, N-acetyl-L-cysteine, DL-homocysteine, L-cysteine methyl ester, L-cysteine ethyl ester, N-carbamoyl cysteine, glutathione or cysteamine.

In one embodiment, the number of cysteine residues on the fusion polypeptide is selected from the group consisting of about 1 to about 12, about 1 to about 10, about 1 to about 8, about 1 to about 6, about 1 to about 4, and about 2 to about 4.

In one embodiment, the method comprises the step of: (b) providing a cleavage reagent capable of cleaving said fusion polypeptide.

In one embodiment, the fusion polypeptide comprises a linker moiety connecting a peptide pair, said linker moiety having a cleavage recognition site for said cleavage reagent.

In one embodiment, the cleavage reagent may be an enzyme. The enzyme may be a protease. The protease may be a restriction protease. The restriction protease may be enterokinase, Factor Xa or thrombin.

In one embodiment, the cleavage reagent may be a chemical reagent. The chemical reagent may be selected from the group consisting of halogenated succinimides, cyanogen halides, hydroxylamines and combinations thereof. Exemplary halogenated succinimides include N-chlorosuccinimide (NCS), N-iodosuccinimide (NIS) and N-bromosuccinimide (NBS). Exemplary cyanogen halides include cyanogen chloride (CNCl), cyanogen iodide (CNI) and cyanogen bromide (CNBr).

In one embodiment, the method comprises the step of: (a3) selecting a peroxide as the oxidant. The step (a3) may comprise the step of: (a4) selecting the peroxide from the group consisting of hydrogen peroxide, an inorganic peroxide, an organic peroxide and perborate salts. Exemplary inorganic peroxides include alkali metal peroxides such as sodium peroxide and potassium peroxide, and alkaline metal peroxides such as calcium peroxide and barium peroxides. Exemplary organic peroxides include diethyl ether peroxide, methyl ethyl ketone peroxide and benzoyl peroxide. Exemplary perborate salts include sodium perborate and calcium perborate.

In one embodiment, the method comprises the step of: (c) maintaining the pH of the reactant solution at a pH of about 3 or higher. Step (c) may comprise the step of: (c1) maintaining the pH of the reactant solution in the range selected from the group consisting of about 6 to about 12, about 7 to about 12, about 8 to about 12, about 9 to about 12, about 10 to about 12, about 6 to about 10, about 6 to about 9, about 6 to about 8, about 4 to about 8, about 4 to about 7, and about 4 to about 6.

In one embodiment, the method comprises the step of: (d) maintaining the temperature of the reactant solution in the range selected from the group consisting of about 2°C to about 42°C, about 10°C to about 42°C, about 20°C to about 42°C, about 30°C to about 42°C, about 2°C to about 40°C, about 2°C to about 30°C, about 2°C to about 20°C, and about 2°C to about 10°C.

In one embodiment, the method comprises the step of: (e) maintaining the initial concentration of the oxidant in the reactant solution at about 50 mM or higher. The oxidant in the reactant solution may be in the range selected from the group consisting of about 50 mM to about 1000 mM, about 100 mM to about 1000 mM, about 200 mM to

about 1000 mM, about 50 mM to about 800 mM, about 50 mM to about 600 mM, about 50 mM to about 400 mM, and about 50 mM to about 200 mM.

According to a second aspect, there is provided a composition for use in a cleavage reaction of a fusion polypeptide, the composition comprising:

(i) a reactant solution of a fusion polypeptide undergoing a cleavage reaction,

(ii) a cleavage reagent capable of cleaving said fusion polypeptide,

(iii) an oxidant capable of increasing the rate of the cleavage reaction, and

(iv) a pH buffer capable of maintaining the pH of the reactant solution at a pH of about 3 or higher.

In one embodiment, the fusion polypeptide comprises at least one sulphur-containing amino acid residue.

In one embodiment, the fusion polypeptide comprises at least one thiol-containing amino acid residue. The thiol-containing amino acid residue may be cysteine or a derivative thereof. The derivative may be D-cysteine, N-acetyl-L-cysteine, DL-homocysteine, L-cysteine methyl ester, L-cysteine ethyl ester, N-carbamoyl cysteine, glutathione or cysteamine.

In one embodiment, the oxidant may be a peroxide. The peroxide may be selected from the group consisting of hydrogen peroxide, an inorganic peroxide, an organic peroxide and perborate salts. Exemplary inorganic peroxides include alkali metal peroxides such as sodium peroxide and potassium peroxide, and alkaline metal peroxides such as calcium peroxide and barium peroxides. Exemplary organic peroxides include diethyl ether peroxide, methyl ethyl ketone peroxide and benzoyl peroxide. Exemplary perborate salts include sodium perborate and calcium perborate.

In one embodiment, the pH buffer may be an acetate buffer, a phosphate buffer, a Tris buffer, a bis-tris buffer or a HEPES buffer.

According to a third aspect, there is provided a kit for use in a cleavage reaction of a fusion polypeptide having at least one sulphur-containing amino acid residue, the kit comprising:

(i) a cleavage reagent capable of cleaving said fusion polypeptide,

(ii) an oxidant capable of increasing the rate of the cleavage reaction, and

(iii) a pH buffer capable of maintaining the pH of a reactant solution of said fusion polypeptide at a pH of about 3 or higher.

Advantageously, the disclosed method provides an efficient, precise and low cost procedure for increasing the reaction rate of fusion polypeptides undergoing a cleavage

reaction. More advantageously, the method can be used in larger scale manufacturing of commercially important recombinant proteins, such as cytokines and vaccines.

DEFINITIONS

This section is intended to provide guidance on the interpretation of the words and phrases set forth below (and where appropriate grammatical variants thereof). Further guidance on the interpretation of certain words and phrases as used herein (and where appropriate grammatical variants thereof) may additionally be found in other sections of this specification.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to any polymer of amino acid residues (dipeptide or greater) linked through peptide bonds or modified peptide bonds and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers. Polypeptides of the present invention include, but are not limited to, naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. The polypeptides of the invention may comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a polypeptide by the cell in which the polypeptide is produced, and will vary with the type of cell. For polypeptides that are made recombinantly, the nature and extent of the modifications in large part will be determined by the post-translational modification capacity of the particular host cell and the modification signals that are present in the amino acid sequence of the polypeptide in question. For instance, glycosylation patterns vary between different types of host cell. Polypeptides are defined herein, in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Proteins may be present as monomeric or as multimeric proteins e.g. as dimers (homo or heterodimers) or trimers.

The term "fusion polypeptide" as used herein, means a polypeptide having a plurality of regions, each corresponding to a distinct peptide or fragment thereof. Fusion polypeptides can include linker moieties connecting the regions thereof. Likewise, the term "fusion protein" as used herein, means a protein having a plurality of regions, each corresponding to a distinct peptide or fragment thereof. Fusion proteins can include linker moieties connecting the regions thereof. Typically, for both fusion polypeptides and fusion proteins, while the plurality of regions are unjoined in their native state, they can be joined by their respective amino and carboxyl termini through a peptide linkage to form a single continuous polypeptide or protein. Plurality in this context means at least two. It will be appreciated that the polypeptide or protein components can be joined directly or joined through a peptide linker(s) as outlined below. In addition, as outlined below, additional components such as fusion partners including protein sorting sequences, etc. may be used. Fusion partners may be included at either the amino or carboxyl termini of the target polypeptide. For example, it is often advantageous to include one or more additional amino acid sequences which may contain secretory or leader sequences, pro-sequences, or sequences which aid in, for instance detection, expression, separation or purification of the protein or to endow the protein with additional properties as desired such as higher protein stability, for example during recombinant production, or for instance to produce an immunomodulatory response. Examples of potential fusion partners include tags, such as polyhistidine tags, epitope tags (short peptide sequences for which a specific antibody is available) and specific binding proteins for aiding purification of the fusion polypeptide, Maltose Binding Protein (MBP) for aiding solubilization of the fusion polypeptide, and Green Fluorescent Protein (GFP) as an indicator for protein folding; enzymes such as ribonuclease S, glutathione S-transferase, beta-galactosidase, luciferase and hemagglutinin; polyethylene glycol; a secretion signal peptide and a label, which may be, for instance, bioactive, radioactive, enzymatic or fluorescent, or an antibody. A fusion polypeptide or fusion protein may be engineered to contain a cleavage site located between the sequence of a fusion partner and the sequence of a target polypeptide / protein so that the fusion partner may be cleaved and purified away from the target polypeptide / protein. The fusion polypeptides or fusion proteins can be cleaved using a cleavage reagent as outlined below. One skilled in the art will be aware that modifications of the sequences of these elements could also be used which would not change the functional properties for which they are used.

The term "cleavage reagent" as used herein refers to a reagent used to cleave the fusion polypeptide to release the target polypeptide from its fusion partner. Suitable cleavage reagents include enzymes, such as proteases, and chemical reagents. The cleavage reagent is selected, taking into consideration the amino acid sequence of the polypeptide to be produced. Care must be taken that, if possible, the recognition sequence of the cleavage reagent for cleavage does not occur in the target polypeptide or elsewhere in the fusion polypeptide other than in the cleavage region. Suitable specifically cleaving proteases are, for example, enterokinase, Factor Xa, thrombin, subtilisin, renin, collagenase, trypsin, chymotrypsin, endoproteinase Lys-C, kallikrein (Carter, P.: In: Ladisch, M. R.; Willson, R. C.; Painton, C. C.; Builder, S. E., eds., Protein Purification: From Molecular Mechanisms to Large-Scale Processes; ACS Symposium Series No. 427, American Chemical Society, pp. 181-193 (1990)), TEV proteinase (Parks, T. D., et al., Anal. Biochem. 216 (1994) 413-417), IgA protease (Pohlner, J., et al., Nature 325 (1987) 458-462), Kex2p proteinase (EP-A 0 467 839) or *S. aureus* V8 proteinase. Suitable specifically cleaving chemical reagents include halogenated succinimides, cyanogen halides, hydroxylamines or combinations thereof. Exemplary halogenated succinimides include N-chlorosuccinimide (NCS), N-iodosuccinimide (NIS) and N-bromosuccinimide (NBS). Exemplary cyanogen halides include cyanogen chloride (CNCl), cyanogen iodide (CNI) and cyanogen bromide (CNBr). Conditions used for cleavage of fusion polypeptides will depend on the cleavage reagent used, and the conditions will be readily apparent to one skilled in the art.

The term "linker" refers to a sequence of amino acids linking regions of a fusion polypeptide, for example a linker may link a fusion partner, such as a tag or motif, to a target polypeptide or peptide. Typically, the linker is not part of the sequence of either the fusion partner or target polypeptide. Typically, the linker is a short sequence of amino acids, for example about one to about 20 amino acids. Typically, the linker moiety includes a cleavage recognition site for the enzymatic or chemical cleavage reagent. The linker moiety may also include additional sequences inserted by one skilled in the art, for example, to provide sufficient spacing between the fusion partner and target polypeptide, or to facilitate cloning.

By "cyclic peptide", "cyclic polypeptide", or "cyclic protein" herein is meant the peptide, polypeptide or protein resulting from cyclization of the peptide backbone.

By "acyclic peptide" or "acyclic polypeptide" is meant linear derivatives of a cyclic peptide or polypetide.

By "recombinant peptide", "recombinant polypeptide" or "recombinant protein" is meant a peptide, polypeptide or protein made using recombinant techniques, i.e., through the expression of a recombinant or synthetic polynucleotide.

By "heterologous polypeptide" or "heterologous protein" herein includes a polypeptide sequence which, in nature, is not expressed by the host organism.

The terms "mutant" and "mutation" include any detectable change in genetic material, e.g. DNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (e.g. DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (e.g. protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., i.e., any kind of mutant.

The terms "permutant" and "permutation" include any arrangement a fusion polypeptide or fusion protein may adopt. For example, an acyclic permutant of a cyclic peptide/protein means a peptide/protein in which the circular backbone of the cyclic peptide/protein has been opened up.

The term "derivative" as used herein, means a chemically or biologically modified version of the compound that is structurally similar to a parent compound. Derivatives of an amino acid include amino acids which are different from naturally-occurring amino acids and which have functions similar to those of the naturally-occurring amino acids. "Naturally-occurring amino acid" as used herein includes alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and other amino acids which may or may not be found in proteins. For example, derivatives of cysteine include D-cysteine, N-acetyl-L-cysteine, DL-homocysteine, L-cysteine methyl ester, L-cysteine ethyl ester, N-carbamoyl cysteine, glutathione and cysteamine.

The terms "purified", "purification" and grammatical variations thereof means that the material in question has been removed from its natural or recombinant host, and associated impurities reduced or eliminated. Preferably, it means an object species is the predominant species present (ie., on a molar basis it is more abundant than any other individual species in the composition), and more preferably a substantially purified fraction is a composition wherein the object species comprises at least about 30 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure

composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to homogeneity (contaminant species cannot be detected in the composition by conventional detection methods such as Coomassie Brilliant Blue staining following SDS-PAGE analysis) wherein the composition consists essentially of a single macromolecular species.

The term "restriction proteases" refers to proteolytic enzymes that cleave polypeptides or proteins at specific recognition sequence sites. The term protease is also used to include the terms peptidase, proteinase, and endopeptidase, which are seen in scientific literature. Proteases can be divided into five different groups, depending on the type of molecule in the groove that carries out the actual work of catalysis: (i) Serine proteases attack the peptide bond of their substrate using the hydroxyl group of the side chain of the amino acid serine, which is present in their catalytic center; (ii) Threonine proteases attack the peptide bond of their substrate using the hydroxyl group of the side chain of the amino acid threonine, which is present in their catalytic center; (iii) Cysteine proteases use the sulphur-hydrogen bond of a cysteine residue to initiate cleavage of the peptide bond; (iv) The acidic carboxyl groups of two aspartyl residues are used in initiating cleavage of peptide bond in aspartyl proteases; and (v) metalloproteases (also known as metalloproteinases) have a tightly bound zinc atom in their catalytic center.

The term "transformation" can be used interchangeably with the term "transfection" when such term is used to refer to the introduction of nucleic acid molecules into cells such as microbial cells, algal cells, bacteria, fungal cells, plant cells or animal cells. Reference to a transformed cell includes a reference to any descendants thereof which also comprise the introduced nucleic acid. Furthermore, variations of the word "transformation", such as "transform" and "transformed", have corresponding meanings.

The term "expression" as used herein refers interchangeably to expression of a gene or gene product, including the encoded protein and/or the expression of a polypeptide or protein on the surface of a cell and/or the expression of a polypeptide or protein that is secreted and released into the culture medium by the cell, as the context requires. Expression of a gene may be determined, for example, by measuring the production of messenger RNA (mRNA) transcript levels. Expression of a polypeptide gene product may be determined, for example, by immunoassay using an antibody(ies) that bind with the polypeptide. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is

inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

By "expression vector" is meant an autonomous genetic element capable of directing the synthesis of a polypeptide encoded by the vector. Such expression vectors are known to one skilled in the art.

The term "cleave" and grammatical variations thereof refers to the cutting of a fusion polypeptide at specific amino acid sequence sites and the term "cleavage" includes scission or proteolysis in this invention.

The term "cleavage reaction" refers to the reaction of a fusion polypeptide in which breakage, either chemical or enzymatic breakage (proteolysis), occurs at one or more amino acid sequence sites of said fusion polypeptide.

The term "catalytic effect", as used herein, refers to any useful effect achieved with addition of an oxidant to a reactant solution containing a fusion polypeptide undergoing a cleavage reaction. A "catalytic effect" may refer to the achievement of the same degree of cleavage within a shorter period of time or using lesser amounts of the cleavage reagent in the presence of an oxidant. For example, a "catalytic effect" may refer to an increase in the reaction rate of a fusion polypeptide undergoing a cleavage reaction in the presence of an oxidant, such as hydrogen peroxide, over the reaction rate of a fusion polypeptide undergoing a cleavage reaction in the absence of said oxidant. The reaction rate may be measured by determining the percentage of the fusion polypeptide that is cleaved after a fixed period of time. The reaction rate in the presence of the oxidant is faster than the reaction rate in the absence of the oxidant. Preferably, the reaction rate in the presence of the oxidant is about 1.5 to about 2 times faster than the reaction rate in the absence of the oxidant. More preferably, the reaction rate in the presence of the oxidant is about 4 to about 5 times faster than the reaction rate in the absence of the oxidant. Most preferably, the reaction rate in the presence of the oxidant is about 8 to about 10 times faster than the reaction rate in the absence of the oxidant.

It will be understood by the skilled person that "molecular weights" measured as described below using electrophoresis are inherently subject to interpretation since they are measured relative to standard molecular weight markers.

"Oxidation" is defined in chemistry as gain of oxygen, loss of hydrogen or loss of electrons. Such oxidation can be carried out using "oxidizing agents" or "oxidants" such as hydrogen peroxide (H_2O_2), inorganic peroxides, organic peroxides and

perborate salts. Exemplary inorganic peroxides include alkali metal peroxides such as sodium peroxide and potassium peroxide and alkaline metal peroxides such as calcium peroxide and barium peroxides. Exemplary organic peroxides include diethyl ether peroxide, methyl ethyl ketone peroxide and benzoyl peroxide. Exemplary perborate salts include sodium perborate and calcium perborate.

A "peroxide" is a chemical substance that contains a peroxo unit, one that has a chemical formula of O_2^{2-}. An example of a peroxide is hydrogen peroxide.

As used herein, a "complete" cleavage reaction includes cleavage reactions where at least 90% of the fusion polypeptides have been cleaved.

As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

As used herein, the term "comprising" means "including." Variations of the word "comprising", such as "comprise" and "comprises," have correspondingly varied meanings. Thus, for example, a composition "comprising" X may consist exclusively of X or may include one or more additional components.

Throughout this disclosure, various aspects of this invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings illustrate a disclosed embodiment and serve to explain the principles of the disclosed embodiment. It is to be understood, however, that

the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Figure 1 shows the nucleic acid and amino acid sequences of kalata B1 and its mutants. (A) The artificial DNA sequence obtained by back translation based on the published amino acid sequence of kalata B1 (GenBank accession No.: 4F393825; GI:15667740). (B) Amino acid sequence of thioredoxin fused with an acyclic peptide of kalata B1. Residues 1 to 109 specifying thioredoxin are italicized. The polyhistidine tag is highlighted in bold. The enterokinase recognition sequence is underlined and the arrow indicates the site of cleavage to yield the 29-amino acid acyclic peptide with no unwanted residues (C). (D) Amino acid sequences of fusion polypeptides for mutants 1a, 2b, 3d, 4d, 5b and 6c. (E) Amino acid sequences of 29-mer target polypeptides of mutants 1a, 2b, 3d, 4d, 5b and 6c.

Figure 2 shows the expression and purification of the recombinant acyclic permutant of kalata B1. (A) SDS-PAGE analysis of the expression level. Lanes 1-3 are the Amersham protein molecular weight marker and the cell extract obtained before and after IPTG induction, respectively. (B) Lanes 1-3 are, respectively, the protein molecular weight standard, the recombinant His_6-tagged thioredoxin-acyclic permutant of kalata B1 fusion polypeptide, and the His_6-tagged thioredoxin as cleavage product. (C) HPLC chromatogram of the acyclic permutant of kalata B1. (D) HPLC chromatogram of mutant 6c of the acyclic permutant of kalata B1.

Figure 3 shows the cleavage of recombinant acyclic permutant of kalata B1 from the fusion polypeptide by enterokinase. (A) 40 μl of reactant solution containing 40 μg of the fusion polypeptide with 0.5 unit of enterokinase was incubated at 25°C. Lanes 3-8 were from samples obtained after 1, 5, 10, 30, 60, and 120 min of incubation, respectively. (B) Same as (A) except that 0.2 M dithiothreitol was included in the reactant solution; (C) Same as (A) but in the presence of 0.2 M hydrogen peroxide.

Figure 4 shows the effect of different concentrations of hydrogen peroxide on the efficiency of enterokinase cleavage. (A) A 30-minute enterokinase reaction: Lanes 1-5 are respectively from enterokinase cleavage reactions containing 0.05, 0.1, 0.15, 0.2 and 0.3 M of hydrogen peroxide, respectively. (B) SDS-PAGE analysis of the effectiveness of enterokinase cleavage in the absence or presence of 0.05M and 0.1M H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers (14300- 220000); Lane 2: Control polypeptide in the absence of H_2O_2 before enterokinase cleavage reaction; Lane 3: Profile of polypeptide molecules after 2 hour cleavage reaction in the absence of H_2O_2; Lane 4:

Control polypeptide with 0.05M H_2O_2 before enterokinase cleavage reaction; Lane 5: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.05M H_2O_2; Lane 6: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 7: Profile of polypeptide molecules after 2 hour cleavage reaction in the absence of H_2O_2; Lane 8: Control polypeptide with 0.1M H_2O_2 before enterokinase cleavage reaction; Lane 9: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.1M H_2O_2. (C) SDS-PAGE analysis of the effectiveness of enterokinase cleavage in the absence or presence of 0.2M and 0.3M H_2O_2. Lane 1: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 2: Profile of polypeptide molecules after 2 hour cleavage reaction without the presence of H_2O_2; Lane 3: Control polypeptide with 0.2M H_2O_2 before enterokinase cleavage reaction; Lane 4: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.2M H_2O_2; Lane 5: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 6: Profile of polypeptide molecules after 2 hour cleavage reaction without the presence of H_2O_2; Lane 7: Control polypeptide with 0.3M H_2O_2 before enterokinase cleavage reaction; Lane 8: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.3M H_2O_2. (D) SDS-PAGE analysis of the effectiveness of enterokinase cleavage in the absence or presence of 0.4M and 0.5M H_2O_2. Lane 1: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 2: Profile of polypeptide molecules after 2 hour cleavage reaction in the absence of H_2O_2; Lane 3: Control polypeptide with 0.4M H_2O_2 before enterokinase cleavage reaction; Lane 4: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.4M H_2O_2; Lane 5: Control polypeptide in the absence of H_2O_2 before enterokinase cleavage reaction; Lane 6: Profile of polypeptide molecules after 2 hour cleavage reaction in the absence of H_2O_2; Lane 7: Control polypeptide with 0.5M H_2O_2 before enterokinase cleavage reaction; Lane 8: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.5M H_2O_2. (E) SDS-PAGE analysis of the effectiveness of enterokinase cleavage in the absence or presence of 0.6M H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers (14 300- 220000); Lane 2: Control polypeptide in the absence of H_2O_2 before enterokinase cleavage reaction; Lane 3: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.6M H_2O_2; Lane 4: Control polypeptide in the absence of 0.6M H_2O_2 before enterokinase cleavage reaction; Lane 5: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.6M H_2O_2. (F) Time course measurements. At each time interval, aliquots of reactant solution was

withdrawn and analysed for the percentage of the cleavage reaction (square, reactant solution containing 0.2 M hydrogen peroxide; triangle, reactant solution using PBS as control).

Figure 5 shows the effect of temperature on the enterokinase cleavage reaction in the absence or presence of hydrogen peroxide. (A) SDS-PAGE analysis of the effectiveness of enterokinase cleavage at 4°C and 20°C in the absence or presence of H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers; Lane 2: Control polypeptide without H_2O_2 before enterokinase cleavage reaction at 4°C; Lane 3: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at 4°C; Lane 4: Control polypeptide with H_2O_2 before enterokinase cleavage reaction at 4°C; Lane 5: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of H_2O_2 at 4°C; Lane 6: Control polypeptide without H_2O_2 before enterokinase cleavage reaction at 20°C; Lane 7: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at 20°C; Lane 8: Control polypeptide with H_2O_2 before enterokinase cleavage reaction at 20°C; Lane 9: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of H_2O_2 at 20°C. (B) SDS-PAGE analysis of the effectiveness of enterokinase cleavage at 37°C and 42°C in the absence or presence of H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers (14 300- 220000); Lane 2: Control polypeptide without H_2O_2 before enterokinase cleavage reaction at 37°C; Lane 3: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at 37°C; Lane 4: Control polypeptide with H_2O_2 before enterokinase cleavage reaction at 37°C; Lane 5: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of H_2O_2 at 37°C; Lane 6: Control polypeptide without H_2O_2 before enterokinase cleavage reaction at 42°C; Lane 7: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at 42°C; Lane 8: Control polypeptide with H_2O_2 before enterokinase cleavage reaction at 42°C; Lane 9: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of H_2O_2 at 42°C.

Figure 6 shows the effect of pH on the enterokinase cleavage reaction in the absence or presence of hydrogen peroxide. (A) SDS-PAGE analysis of the effectiveness of enterokinase cleavage at pH 4 in the absence or presence of H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers (14 300- 220 000); Lane 2: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 3: Profiles of polypeptide molecules after 2 hour cleavage reaction without the presence of H_2O_2; Lane 4: Control polypeptide with 0.3M H_2O_2 before enterokinase cleavage reaction at pH 4.0; Lane 5:

Profiles of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.3M H_2O_2 at pH 4.0. (B) SDS-PAGE analysis of the effectiveness of enterokinase cleavage reaction at pH 7 and pH 10 in the absence or presence of H_2O_2. Lane 1: High-Range Rainbow Molecular weight markers (14 300- 220 000); Lane 2: Control polypeptide without H_2O_2 before enterokinase cleavage reaction; Lane 3: Control polypeptide with 0.3M H_2O_2 before enterokinase cleavage reaction at pH 7.0; Lane 4: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at pH 7.0; Lane 5: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.3M H_2O_2 at pH 7.0; Lane 6: Control polypeptide without H_2O_2 before enterokinase cleavage reaction at pH10.0; Lane 7: Control polypeptide with 0.3M H_2O_2 before enterokinase cleavage reaction at pH 10.0; Lane 8: Profile of polypeptide molecules after 2 hour cleavage reaction without H_2O_2 at pH 10.0; Lane 9: Profile of polypeptide molecules after 2 hour cleavage reaction in the presence of 0.3M H_2O_2 at pH 10.0.

Figure 7 shows the time course data for the acyclic permutant of kalata B1. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 8 shows the time course data for mutant 1a. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 9 shows the time course data for mutant 2b. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 10 shows the time course data for mutant 3d. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 11 shows the time course data for mutant 4d. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 12 shows the time course data for mutant 5b. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

Figure 13 shows the time course data for mutant 6c. (A) 24-hour cleavage reaction in the presence or absence of hydrogen peroxide. (B) 2-hour cleavage reaction in the presence or absence of hydrogen peroxide. (C) SDS-PAGE for reaction without hydrogen peroxide (D) SDS-PAGE for reaction with hydrogen peroxide.

DETAILED DESCRIPTION

The practice of disclosed embodiments will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and recombinant DNA technology which are within the skill of those working in the art. Such techniques are explained fully in the literature. Examples of texts for consultation include the following: Sambrook Molecular Cloning; A Laboratory Manual, Third Edition (2000). For protocols and descriptions of biochemical and biophysical techniques, examples of texts for consultation include those in the series Methods in Enzymology, Academic Press.

All documents cited in this text ("herein cited documents") and all documents cited or referenced in herein cited documents and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety for all purposes. There is no admission that any of the various documents etc. cited in this text are prior art as to the present invention.

Components in the fusion polypeptide for carrying out the methods of the present invention may include: a) a fusion partner; b) a target polypeptide; and c) a linker moiety containing a cleavage recognition site. The fusion partner may include a tag comprising sequences for binding to specific matrices in order to facilitate purification of the fusion polypeptide in a single affinity chromatography step under denaturing or non-denaturing conditions. Tags for aiding solubilization of the fusion polypeptide, for example Maltose Binding Protein (MBP), and tags used as indicators of protein folding, for example Green Fluorescent Protein (GFP), may also be included.

The target polypeptide may be any peptide or polypeptide of interest. For example, the target polypeptide may be a heterologous polypeptide. The number of residues that can

be incorporated into the heterologous polypeptide sequence would be known to one skilled in the art.

The fusion partner(s) may be linked to the target polypeptide at either the amino or carboxyl termini by the linker moiety containing the cleavage recognition site. Restriction proteases or chemical cleavage reagents may be exogenously supplied to the fusion polypeptide in order to cleave the fusion polypeptide into its various components, thereby releasing the target polypeptide of interest.

The method of increasing the reaction rate of a fusion polypeptide undergoing a cleavage reaction according to the invention may advantageously be incorporated in the production of a recombinant polypeptide or protein. Typically in such circumstances, steps leading to the method of the present invention of cleaving the fusion polypeptide to release the target polypeptide may include:

a) preparing a recombinant vector;

b) transforming a host cell with the recombinant vector;

c) cultivating the obtained transformant;

d) collecting the cultured product containing the fusion polypeptide; and

e) purifying the fusion polypeptide from the cultured product.

Any expression vector suitable for expression of the gene of interest can be used. Examples of vectors include the pET series from Novagen, the pQE series from Qiagen, and pBR322 in the case in which *Escherichia coli* serves as a host and pUB110 in the case in which *Bacillus subtilis* serves as a host. The lac or tac promoter, commonly used for inducing the expression of fused or unfused gene products, can be inserted into the vector. Alternatively, the T7 promoter can be used. A sequence encoding a fusion partner containing a tag may be cloned downstream of and in operable linkage with the promoter. This sequence contains the translational start codon ATG. The sequence coding for the cleavage recognition site can be inserted between the fusion partner and target polypeptide encoding sequences. Multiple restriction sites for the insertion of the target gene can be present downstream of the fusion partner. It is preferable to maintain the number of sites in the multiple cloning region (MCR) to a minimum. A nucleotide sequence coding for a translation termination codon, in all 3 reading frames, can be inserted downstream of the target polypeptide encoding sequence. A sequence encoding the target polypeptide of interest can be inserted in the multiple cloning site in the appropriate reading frame. The resulting construct can be introduced into an appropriate host cell. A host is transformed by use of the thus-obtained recombinant vector through conventional methods such as the

protoplast method or the competent cell method. Although no particular limitation is imposed on the host, microorganisms are preferred. Examples include Gram-positive bacteria such as microorganisms belonging to the genus *Bacillus*, Gram-negative bacteria such as *Escherichia coil,* yeast belonging to *Saccharomyces*, and fungus belonging to *Aspergillus*.

The strains are inoculated in a medium containing an assimilable carbon source, a nitrogen source and essential nutrients, and are cultured through conventional fermentation methods.

Collection and purification of a target polypeptide from the thus-obtained culture broth can be performed according to conventional methods applicable to the collection and purification of common proteins. For example, cells are separated from the culture broth by centrifugation or filtration, and the target polypeptide can be obtained from the supernatant through conventional purification procedures, examples of which include: fractionation on an ion-exchange column; ethanol precipitation; affinity chromatography; ultracentrifugation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind polyhistidine-tagged forms of polypeptides. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, *Methods in Enzymology,* 182 (1990) and Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular polypeptide produced.

The transformed cells, after lysis, and fractionation into the soluble and the insoluble fractions, can be analyzed to determine the solubility of the fusion polypeptide. When the fusion polypeptide is produced in an insoluble form, it is present in the form of inclusion bodies. The insoluble pellet which contains the inclusion bodies is washed in a buffer containing sodium phosphate, DPT, and Triton X-100. The contaminating components are thereby removed, leaving the inclusion bodies containing the target polypeptide in the insoluble pellet.

If the fusion polypeptide is present in the soluble fraction (aggregated or non-aggregated form), then the full-length polypeptide can be purified using affinity column chromatography, depending on the type of purification tag in the fusion partner fused to

the target polypeptide. The cleavage of purified fusion polypeptides with the restriction proteases or chemical cleavage reagents is conducted by dissolving the fusion polypeptide, e.g., in a suitable buffer such as Tris buffer or phosphate buffer (e.g., especially at a pH of 6-9), depending on the solubility properties of the particular fusion polypeptide involved. The cleavage reagent(s) and oxidant are added to the solution and incubation ensues at a temperature and time optimized, e.g., by routine, conventional experiments, generally 25°C-40°C, for 0.5-10 hours.

EXAMPLES

Materials and methods

Synthetic polypeptides and oligonucleotides

All oligonucleotides used for plasmid construction were purchased from Proligo (Singapore). *Escherichia coli* strain DH5α (Stratagene, La Jolla, California, USA) was used as the host for all cloning procedures while expression of the synthetic fusion polypeptide was achieved using *E. coli* strain BL21 (DE3) (Novagen, Madison, Wisconsin, USA) as host.

Construction of expression vectors

An oligonucleotide containing the coding sequence of the acyclic permutant of kalata B1 (Fig. 1A) was artificially synthesized with 13 silent codon changes made according to *E. coli* codon preference. To facilitate directional cloning into the bacterial expression vector, pET-32a (Novagen), 5' *Bgl* II and 3' *Sal I* sites were incorporated by PCR amplification of the synthetic DNA. The 5' primer also contained a 15-bp sequence encoding an enterokinase recognition site downstream of the *BgII* restriction recognition sequence. Directional insertion of the amplified product places the kalata Bl-coding sequence downstream of the thioredoxin. The resulting amino acid sequence of the expressed fusion polypeptide is shown in Fig. 1B. The incorporated enterokinase recognition site allows separation of the acyclic polypeptide from the fusion partner without leaving any unwanted residues on the 29-mer polypeptide (Fig. 1C).

Mutants of the acyclic permutant of kalata B1

Six mutants, labelled 1a, 2b, 3d, 4d, 5b and 6c, with amino acid substitutions were similarly produced. The amino acid sequences of the expressed fusion polypeptides of

these mutants are shown in Fig. 1D while their corresponding acyclic 29-mer polypeptides after cleavage are shown in Fig 1E.

Fusion polypeptide expression and purification

An overnight culture of the transformed *E. coli* strain BL21 (DE3) with the expression vector was diluted in a ratio of 1:25 into 100 ml of growth medium (LB medium containing 100 ug/ml of ampicillin). The culture was incubated at 37°C until its optical density (OD) at 600nm reaches 0.6. The polypeptide expression was induced by the addition of 0.1 mM isopropyl-1-thiol-p-D-galactoside (IPTG) for 3 h. The cell pellet was harvested and resuspended in PBS buffer. After sonication, the resulting cell lysate was cleared of bacterial debris by ultracentrifugation. The fusion polypeptide with a His_6-tagged thioredoxin was absorbed onto a Nickel-charged metal affinity column made from chelating sepharose (Qiagen, Germany). The polypeptide was eluted from the column using 300 mM of imidazole. The purity and quantity of the fusion polypeptide was examined by SDS-PAGE and using a BCA (bicinchoninic acid) protein assay kit (Sigma). For the SDS-PAGE analysis, the fusion polypeptide was electrophoresed on a 12% SDS-polyacrylamide gel and quantitatively analyzed using a BioRad GS-800 scanning densitometer.

Release of the target polypeptide upon enterokinase cleavage

To remove imidazole, the fusion polypeptide preparation obtained from metal chelating affinity chromatography was subjected to buffer change to PBS buffer using Vivaspin ultrafiltration spin columns with a molecular weight cut-off of 30,000 (Vivascience, Germany). The resulting polypeptide solution was treated with enterokinase to release the 29-mer recombinant polypeptide. For a standard cleavage reaction in this study, 40 µl of reactant solution containing 40 µg of the fusion polypeptide with 0.5 unit of enterokinase (Novagen) was incubated at 25 °C overnight. The efficiency of the cleavage reaction was monitored by SDS-PAGE analysis by monitoring the densitometric changes of the fusion polypeptide band at 22 kDa. The target polypeptide with a molecular weight of 2.9 kDa in size could not be visualized in conventional SDS-PAGE. However, it could easily be detected and analyzed with reversed-phase HPLC. Reversed-phase high performance liquid chromatography (RP-HPLC) was carried out on a Shimadzu LC-10Avp serious apparatus with a UV detector and a VP-ODS CIS column (250.0 x 4.6 mm, 5 µm, 300A). A gradient was achieved using buffer A (containing

0.05% trifluoroacetic acid in water) and buffer B (containing 90% acetonitrile and 0.05% trifluoroacetic acid in water). The flow rate was 1 ml/min with buffer B being increased from 0% to 90% in 40 minutes. The chromatograms were recorded at a wavelength of 225 nm and the data analysed using LCsolution Version 1.0 (Shimadzu).

Effect of hydrogen peroxide on enterokinase cleavage

To study the effect of hydrogen peroxide on enterokinase cleavage, different concentrations of hydrogen peroxide was included in the reactant solution at different pHs and temperatures. Its effect on cleavage was monitored by the separation of the His_6-tagged thioredoxin-acyclic permutant of kalata B1 fusion polypeptide from one of its cleavage products, the His_6-tagged thioredoxin, on SDS-PAGE gels. Where time course measurements and calculation of the percentage of the fusion polypeptide cleavage were performed, aliquots were removed at various time intervals from a reactant solution of 80 µl in volume containing 60 µg of the fusion polypeptide with 1 unit of enterokinase (Novagen). The reaction was carried out at 25°C unless otherwise specified. The product profile in each aliquot was examined on SDS-PAGE after denaturation with sample buffer containing 1% SDS and heating at 95 °C for 5 min. After electrophoresis, the SDS-PAGE gels were dried. The cleavage profile and percentage of the fusion polypeptide cleaved was quantitatively analyzed using BioRad GS-800 scanning densitometer.

Results and discussion

Expression and purification of thioredoxin-target peptide fusion polypeptide

Based on back translation tools, a synthetic codon-optimised cDNA sequence encoding the 29-mer acyclic permutant of a plant cyclopeptide was designed. This DNA fragment was synthesized and fused in-frame to His_6-tagged thioredoxin in the bacterial expression vector, pET32a. Upon induction with IPTG, the fusion polypeptide was overexpressed in the bacterial host, *Escherichia coli* strain BL21(DE3). The fusion polypeptide is in the cytoplasm as a soluble polypeptide with an apparent molecular weight of 22 kDa based on SDS-PAGE analysis (Fig. 2A and 2B). Scanning densitometric analysis revealed that the fusion polypeptide was overexpressed at a level that accounts for approximately 30% of total cellular proteins. We could obtain up to 20 mg of soluble fusion polypeptide from 1 litre of bacterial culture. Optimal expression of the fusion polypeptide was achieved when the culture was carried out at 37°C and the induction was performed with IPTG at a concentration of 0.1 M.

To purify the fusion polypeptide, metal chelating sepharose column was employed to remove cellular proteins, while the absorbed His_6-tagged thioredoxin-acyclic permutant of kalata B1 fusion polypeptide was eluted from the column using imidazole (300mM). The purity and identity of the polypeptide was checked by polyacrylamide gel electrophoresis and staining using Pierce GelCode blue stain reagent. The yield and concentration of the polypeptide preparation was examined based on BCA protein assay method. Typically, a polypeptide solution with purity of over 95% and final concentration of 2 to 3 mg/ml in PBS buffer can be obtained by using Vivaspin ultrafiltration columns for concentration and buffer exchange.

Enterokinase cleavage of the fusion polypeptide

To facilitate the purification of the target polypeptide from the His_6-tagged thioredoxin, an enterokinase recognition sequence was introduced immediately upstream of the target polypeptide, allowing the latter to be released without leaving any unwanted residues upon treatment with enterokinase. To achieve maximal release of the target polypeptide, the cleavage reaction is typically performed overnight at 25°C. The efficiency of the cleavage reaction was monitored by SDS-PAGE analysis by observing the disappearance of the fusion polypeptide band at 22 kDa. The target polypeptide with molecular weight of 2.9 kDa in size could not be detected in conventional SDS PAGE analysis. However, it could easily be monitored on reversed-phase HPLC. For example, Fig. 2C shows the acyclic permutant of kalata B1 eluting at about 30 minutes while Fig. 2D shows one of its mutants, mutant 6c, eluting at about 29 minutes.

Effect of oxidizing and reducing agents

An oxidative agent (hydrogen peroxide) or a reducing agent (DTT) was alternately included in the reactant solution to examine if any one of them plays a role in the cleavage reaction. It was observed that while no significant effect was observed with addition of DTT at a concentration of 10-200 mM, the presence of hydrogen peroxide did have an effect on the rate of the cleavage reaction. By monitoring the densitometric change of the band at 22 kDa on SDS-PAGE, representing the intact His_6-tagged thioredoxin-acyclic permutant of kalata B1 fusion polypeptide, it was observed that more than 95% of cleavage took place with the reactant solution containing 0.2 M hydrogen peroxide (Fig. 3C). On the other hand, only 25% of the fusion polypeptide was cleaved when the reaction

was carried out in the absence of hydrogen peroxide (Fig. 3A) or in the presence of 0.2M DTT (Fig. 3B).

Effect of hydrogen peroxide on the enterokinase cleavage reaction

(a) Effect of different concentrations of hydrogen peroxide

It was observed that the concentration of the hydrogen peroxide in the reactant solution has an effect on the enterokinase cleavage reaction. Fig. 4A shows the results of 30-min enterokinase cleavage reactions at different concentrations of hydrogen peroxide. With an increase in the concentration of hydrogen peroxide from 0.05 to 0.3M, there was a gradual improvement in the cleavage efficiency. Two-hour cleavage reactions with different concentrations of hydrogen peroxide were also carried out (Figs. 4B-E) with the results summarized in Table 1. In a 2-hr reaction, 0.2 M hydrogen peroxide was sufficient to achieve 100% cleavage of the fusion polypeptide. Furthermore, when higher concentrations of hydrogen peroxide (>0.5M) were used, we noticed there are multiple bands generated from the cleavage reaction (Figs. 4D and E). It is likely that hydrogen peroxide at a concentration higher than 0.5 M causes oxidative damage to enterokinase, thereby affecting its specificity and activity (Li et al., Biochemistry (Mosc) 60(10) (2004) 1136-1142).

Table 1. Quantification and comparison of the efficiency of enterokinase cleavage reaction in terms of the percentage of the polypeptide cleaved at different concentrations of hydrogen peroxide

	0.05M	0.1M	0.2M	0.3M	0.4M	0.5M	0.6M
Control polypeptide without H_2O_2 (0 hour)	0	0	0	0	0	0	0
Control polypeptide in the presence of H_2O_2 (0 hour)	0	0	0	0	0	0	0
2 hour cleavage reaction without H_2O_2	58.2	62.3	63.6	61.1	60.4	60.3	62
2 hour cleavage reaction in the presence of H_2O_2	63.6	96.5	100	100	100	100	100

The time course measurements (refer to Fig. 4F) indicate that it only took 30 minutes for more than 95% of the fusion polypeptide to be cleaved in the presence of 300 mM of hydrogen peroxide. On the contrary, only 25% cleavage took place when the reaction was carried out in the absence of hydrogen peroxide in the same time interval. In fact, it normally takes an overnight incubation for the cleavage to be more than 90% complete in the absence of hydrogen peroxide. It is still not known how hydrogen peroxide is involved in the enterokinase cleavage reaction.

(b) Effect of different temperatures

The effect of temperature on the enterokinase cleavage reaction was also studied (Figs. 5A and B). In a 2-hr reaction carried out at 4, 20, 37, and 42°C, respectively, the presence of hydrogen peroxide was seen to have a catalytic effect on the enterokinase cleavage reaction at each and every temperature (Table 2). Advantageously in the presence of hydrogen peroxide, enterokinase cleavage can proceed to 84% completion in a 2-hr reaction even at 4°C. The presence of hydrogen peroxide therefore enables efficient cleavage of thermally unstable polypeptides / proteins at a temperature that is not harmful to the target polypeptide / protein.

Table 2. Quantitation and comparison of the efficiency of enterokinase cleavage reaction in terms of the percentage of the polypeptide cleaved at different temperatures

	4°C	20°C	37°C	42°C
Control polypeptide without H_2O_2	0	0	0	0
Control polypeptide in the presence of H_2O_2	0	0	0	0
2 hour cleavage reaction without H_2O_2	10.1	37.2	66.3	75.7
2 hour cleavage reaction in the presence of H_2O_2	83.6	84.3	100	100

(c) Effect of different pHs

The effect of pH on the enterokinase cleavage reaction was studied (Figs. 6A and B). In a 2-hr reaction, it was observed that hydrogen peroxide was able to catalyse the enterokinase cleavage under acidic conditions (pH 4.0), neutral conditions (pH 7.0) and alkaline conditions (pH 11.0). It was further observed that conducting the reaction at pH 7.0 and 10.0 in the presence of 0.3 M hydrogen peroxide enabled achievement of 100% cleavage of the fusion polypeptide in a 2-hr reaction (Table 3).

Table 3. Quantitation and comparison of the efficiency of enterokinase cleavage reaction in terms of the percentage of the polypeptide cleaved

	pH 4	pH 7	pH 10
Control polypeptide without H_2O_2	0	0	0
Control polypeptide in the presence of H_2O_2	0	0	0
2 hour cleavage reaction without H_2O_2	28.7	71.3	67.6
2 hour cleavage reaction in the presence of H_2O_2	89.1	100	100

(d) Effect of hydrogen peroxide on cleavage of different mutants of the acyclic permutant of kalata B1

To demonstrate that the catalytic effect of hydrogen peroxide on the enterokinase cleavage system can be applied to a wider variety of peptides, time course experiments were carried out on the acyclic permutant of kalata B1 and its six mutants

(Figs. 7-13). The same observation can be made in that hydrogen peroxide greatly enhanced the enterokinase cleavage rate of all seven fusion polypeptides.

It was observed that the presence of hydrogen peroxide in the cleavage reaction accelerated the rate of enterokinase cleavage. With the addition of hydrogen peroxide into enterokinase reactant solution, the release of the target polypeptide was complete within 1 hour in some cases, which is in contrast to the 24 hours if hydrogen peroxide is not included in the reaction.

The above assay was applied to 7 fusion polypeptides with different sequences, which can be shown in Figs. 7-13, that hydrogen peroxide can greatly enhance/catalyse the enterokinase cleavage reaction.

With the acyclic permutant of kalata B1, more than 90% of the fusion polypeptide was cleaved after a 2 hour reaction in the presence of hydrogen peroxide (Figure 7). In contrast, only 57.3% of cleavage was observed when no hydrogen peroxide was added.

Likewise, as illustrated in Fig. 8, addition of hydrogen peroxide to the reactant solution resulted in 90.1% of mutant 1a being cleaved in 2 hours while cleavage in the absence of hydrogen peroxide only resulted in 56.9% cleavage of the mutant.

A similar catalytic effect of hydrogen peroxide was seen in the cleavage of mutant 2b, where 94.7% of the fusion polypeptide was cleaved in 2 hours when hydrogen peroxide was added and only 54.6% of the fusion polypeptide was cleaved when no hydrogen peroxide was added (Fig. 9).

With mutant 3d, although only 70.5% of the fusion polypeptide was cleaved in a 2-hour reaction in the presence of hydrogen peroxide, this was still markedly higher than the 28.2% cleavage observed in the absence of hydrogen peroxide (Fig. 10).

Where hydrogen peroxide was added to the reactant solutions containing mutants 4d, 5b or 6c, more than 80% cleavage of the respective fusion polypeptides was observed after a 2-hour reaction (Figs. 11-13). In contrast, only 52.1-59.7% cleavage of the respective fusion polypeptides were seen with the three mutants when no hydrogen peroxide was added.

Although the extent of the enhancement varies depending on the nature and structure of the polypeptide or protein, the catalysis might be universal as far as the enterokinase is applied in the cleavage reaction. Thus, in view of the above, the present invention offers a simple and cost-effective method for accelerating cleavage of fusion polypeptides.

Applications

Advantageously, the disclosed method accelerates cleavage of fusion polypeptides. Accordingly, embodiments of the disclosed method overcomes the disadvantages of the prior art. For example, the disclosed methods could be used to efficiently and precisely cleave fusion polypeptides to release a target polypeptide. Accordingly, the disclosed method could be used in the production of pharmaceutical peptides/proteins where a desired peptide/protein sequence is substantially free of additional amino acid residues which affect the structural, physical and functional properties of the peptides/proteins.

It will also be appreciated that because, as it has surprisingly been discovered, the addition of a peroxide to the reactant mixture increases the cleavage reaction rate, it is not necessary to use higher enzyme quantities or higher chemical reagent concentrations to increase rate of reaction. This is beneficial considering the relatively low cost of peroxides in comparison to the cost of enzymatic or chemical cleavage reagents. The method therefore reduces costs and avoids unwanted cleavage at secondary cleavage sites.

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

CLAIMS

1. A method of increasing the reaction rate of a fusion polypeptide undergoing a cleavage reaction, the method comprising the step of:

(a) providing an oxidant to a reactant solution of the fusion polypeptide as it undergoes the cleavage reaction to increase the rate of said reaction.

2. A method as claimed in claim 1, wherein step (a) comprises the step of:

(a1) providing the fusion polypeptide having at least one sulphur-containing amino acid residue.

3. A method as claimed in claim 1, wherein step (a) comprises the step of:

(a2) providing the fusion polypeptide having at least one thiol-containing amino acid residue.

4. A method as claimed in claim 3, wherein the thiol-containing amino acid residue is cysteine or a derivative thereof.

5. A method as claimed in claim 4, wherein the derivative is selected from the group consisting of D-cysteine, N-acetyl-L-cysteine, DL-homocysteine, L-cysteine methyl ester, L-cysteine ethyl ester, N-carbamoyl cysteine, glutathione and cysteamine.

6. A method as claimed in claim 1, comprising the step of:

(b) providing a cleavage reagent capable of cleaving said fusion polypeptide.

7. A method as claimed in claim 6, wherein the fusion polypeptide comprises a linker moiety connecting a peptide pair, said linker moiety having a cleavage recognition site for said cleavage reagent.

8. A method as claimed in claim 6, wherein step (b) comprises the step of:

(b1) providing an enzyme as the cleavage reagent.

9. A method as claimed in claim 8, wherein the enzyme is a protease.

10. A method as claimed in claim 9, wherein the protease is a restriction protease.

11. A method as claimed in claim 10, wherein the restriction protease is selected from the group consisting of enterokinase, Factor Xa and thrombin.

12. A method as claimed in claim 6, wherein step (b) comprises the step of:

(b2) providing a chemical reagent as the cleavage reagent.

13. A method as claimed in claim 12, wherein the chemical reagent is selected from the group consisting of halogenated succinimides, cyanogen halides, hydroxylamines and combinations thereof.

14. A method as claimed in claim 1, comprising the step of:

(a3) selecting a peroxide as the oxidant.

15. A method as claimed in claim 14, comprising the step of:

(a4) selecting the peroxide from the group consisting of hydrogen peroxide, an inorganic peroxide, an organic peroxide and perborate salts.

16. A method as claimed in claim 1, comprising the step of:

(c) maintaining the pH of the reactant solution at a pH of about 3 or higher.

17. A method as claimed in claim 16, wherein step (c) comprises the step of:

(c1) maintaining the pH of the reactant solution in the range selected from the group consisting of about 6 to about 12, about 7 to about 12, about 8 to about 12, about 9 to about 12, about 10 to about 12, about 6 to about 10, about 6 to about 9, about 6 to about 8, about 4 to about 8, about 4 to about 7, and about 4 to about 6.

18. A method as claimed in claim 1, comprising the step of:

(d) maintaining the temperature of the reactant solution in the range selected from the group consisting of about 2°C to about 42°C, about 10°C to about 42°C, about 20°C to about 42°C, about 30°C to about 42°C, about 2°C to about 40°C, about 2°C to about 30°C, about 2°C to about 20°C, and about 2°C to about 10°C.

19. A method as claimed in claim 1, comprising the step of:

(e) maintaining the concentration of the oxidant in the reactant solution at about 50 mM or higher.

20. A method as claimed in claim 19, comprising the step of:

(e1) maintaining the concentration of the oxidant in the reactant solution in the range selected from the group consisting of about 50 mM to about 1000 mM, about 100 mM to about 1000 mM, about 200 mM to about 1000 mM, about 50 mM to about 800 mM, about 50 mM to about 600 mM, about 50 mM to about 400 mM, and about 50 mM to about 200 mM.

21. A composition for use in a cleavage reaction of a fusion polypeptide, the composition comprising:

(i) a reactant solution of a fusion polypeptide undergoing a cleavage reaction,

(ii) a cleavage reagent capable of cleaving said fusion polypeptide,

(iii) an oxidant capable of increasing the rate of the cleavage reaction, and

(iv) a pH buffer capable of maintaining the pH of the reactant solution at a pH of about 3 or higher.

22. A composition as claimed in claim 21, wherein the fusion polypeptide has at least one sulphur-containing amino acid residue.

23. A composition as claimed in claim 21, wherein the fusion polypeptide has at least one thiol-containing amino acid residue.

24. A composition as claimed in claim 23, wherein the thiol-containing amino acid residue is cysteine or a derivative thereof.

25. A composition as claimed in claim 24, wherein the derivative is selected from the group consisting of D-cysteine, N-acetyl-L-cysteine, DL-homocysteine, L-cysteine methyl ester, L-cysteine ethyl ester, N-carbamoyl cysteine, glutathione and cysteamine.

26. A composition as claimed in claim 21, wherein the cleavage reagent is an enzyme.

27. A composition as claimed in claim 26, wherein the enzyme is a protease.

28. A composition as claimed in claim 27, wherein the protease is a restriction protease.

29. A composition as claimed in claim 28, wherein the restriction protease is selected from the group consisting of enterokinase, Factor Xa and thrombin.

30. A composition as claimed in claim 21, wherein the cleavage reagent is a chemical reagent.

31. A composition as claimed in claim 30, wherein the chemical reagent is selected from the group consisting of halogenated succinimides, cyanogen halides, hydroxylamines and combinations thereof.

32. A composition as claimed in claim 21, wherein the oxidant is a peroxide.

33. A composition as claimed in claim 32, wherein the peroxide is selected from the group consisting of hydrogen peroxide, an inorganic peroxide, an organic peroxide and perborate salts.

34. A composition as claimed in claim 21, wherein the pH buffer is selected from the group consisting of acetate buffers, phosphate buffers, Tris buffers, bis-tris buffers or HEPES buffers.

35. A kit for use in a cleavage reaction of a fusion polypeptide having at least one sulphur-containing amino acid residue, the kit comprising:

(i) a cleavage reagent capable of cleaving said fusion polypeptide,

(ii) an oxidant capable of increasing the rate of the cleavage reaction, and

(iii) a pH buffer capable of maintaining the pH of the reactant solution of said fusion polypeptide at a pH of about 3 or higher.

36. A kit as claimed in claim 35, wherein the cleavage reagent is an enzyme.

37. A kit as claimed in claim 36, wherein the enzyme is a protease.

38. A kit as claimed in claim 37, wherein the protease is a restriction protease.

39. A kit as claimed in claim 38, wherein the restriction protease is selected from the group consisting of enterokinase, Factor Xa and thrombin.

40. A kit as claimed in claim 35, wherein the cleavage reagent is a chemical reagent.

41. A kit as claimed in claim 40, wherein the chemical reagent is selected from the group consisting of halogenated succinimides, cyanogen halides, hydroxylamines and combinations thereof.

42. A kit as claimed in claim 35, wherein the oxidant is a peroxide.

43. A kit as claimed in claim 42, wherein the peroxide is selected from the group consisting of hydrogen peroxide, an inorganic peroxide, an organic peroxide and perborate salts.

44. A kit as claimed in claim 35, wherein the pH buffer is selected from the group consisting of acetate buffers, phosphate buffers, Tris buffers, bis-tris buffers or HEPES buffers.