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(54) Title: ACTIVATION OF ZYMOGENS BY IMMOBILIZED PROTEASE ENZYMES

(57) Abstract: Methods for activating zymogens of transglutaminases involving the use of at least one immobilized protease are disclosed as well as activated transglutaminases made by such methods.

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ACTIVATION OF ZYMOGENS BY IMMOBILIZED PROTEASE ENZYMES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 63/159,313, filed March 10, 2021, which is incorporated herein by reference in its entirety.

FIELD

[0002] Activation of zymogens, in particular, use of immobilized proteases to activate zymogens is disclosed.

BACKGROUND

[0003] Immobilized enzymes are typically more robust and more resistant to environmental changes compared to free enzymes in solution. More importantly, the heterogeneity of immobilized enzyme systems allows an easy or more simplified recovery of both enzymes and products, multiple re-use of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs.

SUMMARY

[0004] In a first embodiment, there is disclosed a method for activating a zymogen of a transglutaminase (Tgase), said method comprising contacting the zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.

[0005] In a second embodiment, there is disclosed an activated transglutaminase produced by contacting a zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.

[0006] In a third embodiment, the transglutaminase is a microbial transglutaminase.

[0007] In a fourth embodiment, the microbial transglutaminase is a *Streptomyces mobaraensis* transglutaminase or a variant thereof.

[0008] In a fifth embodiment, the at least one immobilized protease is selected from the group consisting of bacterial proteases and fungal proteases.

[0009] In a sixth embodiment, the bacterial protease is subtilisin.

[0010] In a seventh embodiment, the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 shows SDS-PAGE analysis of *S. mobarensis* Tgase, as described in Example 2. Lane 1 – mature Tgase; Lane 2 – zymogen (pro-Tgase); Lane 3 – clarified lysate containing zymogen (crude pro-Tgase); Lane 4 - clarified lysate treated with immobilized protease for 60 minutes; Lane L - protein ladder. Expected molecular weight of the zymogen (pro-Tgase) is 43.6 kDa and the expected molecular weight of the mature Tgase is 38.9 kDa.

DETAILED DESCRIPTION

[0012] All patents, patent applications, and publications cited herein are incorporated by reference in their entireties.

[0013] In this disclosure, many terms and abbreviations are used. The following definitions apply unless specifically stated otherwise.

[0014] As used herein, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

[0015] The terms "and/or" and "or" are used interchangeably herein and refer to a specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used a phrase such as "A, B and/or C" is intended to encompass each of the following aspects: A, B and C; A, B or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0016] Words using the singular include the plural, and vice versa, unless the context clearly dictates otherwise.

[0017] The terms "comprises," "comprising," "includes," "including," "having" and their conjugates are used interchangeably and mean "including but not limited to." It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0018] The term "consisting of" means "including and limited to."

[0019] The term "consisting essentially of" means the specified material of a composition, or the specified steps of a methods, and those additional materials or steps that do not materially affect the basic characteristics of the material or method.

[0020] “Optional” or “optionally” means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

[0021] Throughout this application, various embodiments 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 embodiments described herein. 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 subranges such as from 1 to 2, from 1 to 3, from 1 to 4 and from 1 to 5, from 2 to 3, from 2 to 4, from 2 to 5, from 2 to 6, from 3 to 4, from 3 to 5, 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.

[0022] The term “about” as used herein can allow for a degree of variability in a value or range, for example, within 10%, within 5%, or within 1% of a stated value or of a stated limit of a range.

[0023] The terms “zymogen”, “proprotein”, and “proenzyme”, are used interchangeably herein, and refer to an inactive precursor of an enzyme, which may be converted into an active/activated or mature enzyme by catalytic action, such as via proteolytic cleavage of a pro-sequence.

[0024] A “pro-sequence” refers to a polypeptide sequence within an expressed protein, e.g., a zymogen, which is typically cleaved from the protein to produce an active protein, such as an enzyme. In some embodiments, a pro-sequence may be essential for correct folding of the protein. In some embodiments, cleavage of the pro-sequence results in transition of an inactive enzyme to active or activated enzyme.

[0025] The terms “mature”, “active”, and “activated” are used interchangeably herein. An activated form of a protein, polypeptide, or peptide refers to the functional form of the protein, polypeptide, or enzyme without a signal, silencing, or chaperoning propeptide sequence. Additionally, the mature enzyme may be truncated or extended relative to the mature sequence while maintaining the desired catalytic activity (e.g., cross-linking activity).

[0026] Transglutaminases (Tgase, EC2.3.2.13) are a family of enzymes that catalyze the formation of an isopeptide bond between a primary amine, for example, the ϵ -amine of a lysine molecule, and the acyl group of a protein- or peptide-bound glutamine.

Transglutaminases may catalyze a transamidation reaction between glutamyl and lysyl side

chains of target proteins. Proteins possessing Tgase activity have been found in microorganisms, plants, and animals. Tgases are widely distributed in various organs, tissues, and bodily fluids. Tgases also form extensively cross-linked, generally insoluble, protein biopolymers that are needed for an organism to create barriers and stable structures. As used herein, an "activated Tgase" is a Tgase having or capable of reacting with amino acids, peptides and/or proteins.

[0027] In contrast to eukaryotic Tgases, Tgases of microbial origin are calcium-independent, which represents a major advantage for their practical use. "Microbial transglutaminase" (Tgase, EC 2.3.2.13) is one of the most extensively studied industrial enzymes for protein functionalization and protein cross-linking because of its ability to polymerize or functionalize proteins through the formation of a stable ϵ -(γ -glutamyl)lysine isopeptide bond without the constraint of a consensus sequence or additional cofactors.

[0028] An "immobilized protease" refers to immobilization of a protease to/on a matrix or support.

[0029] A "protease" (also called a peptidase or proteinase) refers to enzymes capable of cleaving peptide bonds. Proteases are any of various enzymes, such as endopeptidases and exopeptidases, that catalyze the hydrolytic breakdown of proteins into peptides and amino acids. Proteases can be classified into seven broad groups: serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, metalloproteases, and asparagine peptide lyases. Proteases can be found in animals, plants, bacteria, fungi, archaea, and viruses. The terms "protease", "peptidase", and "proteinase" are used interchangeably herein.

[0030] "Subtilisin" is a protease initially obtained from *Bacillus subtilis*.

[0031] The terms "peptides", "proteins", and "polypeptides" are used interchangeably herein and refer to a polymer of amino acids joined together by peptide bonds. A "protein" or "polypeptide" comprises a polymeric sequence of amino acid residues. The single and 3-letter code for amino acids as defined in conformity with the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), well known to those skilled in the art, is used throughout this disclosure. The single letter X refers to any of the twenty amino acids. It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code. Mutations can be named by the one letter code for the parent amino acid, followed by a position number and then the one letter code for the variant amino acid. For example, mutating glycine (G) at position 87 to serine (S) is represented as "G087S" or "G87S". When describing modifications, a position followed by amino acids listed in parentheses indicates a list of substitutions at that position by any of

the listed amino acids. For example, 6(L, I) means position 6 can be substituted with a leucine or isoleucine. At times, in a sequence, a slash (/) is used to define substitutions, e.g., F/V, indicates that the position may have a phenylalanine or valine at that position.

[0032] The term "amino acid" refers to the basic chemical structural unit of a protein, peptide, or polypeptide.

[0033] As used herein, with regard to amino acid residue positions, "corresponding to" or "corresponds to" or "correspond to" or "corresponds" refers to an amino acid residue at the enumerated position in a protein or peptide, or an amino acid residue that is analogous, homologous, or equivalent to an enumerated residue in a protein or peptide. As used herein, "corresponding region" generally refers to an analogous position in a related protein or a reference protein.

[0034] One of ordinary skill in the art will appreciate that modifications of amino acid sequences disclosed herein can be made while retaining the function associated with the disclosed amino acid sequences. For example, it is well known in the art that alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded protein, are common.

[0035] Related (and derivative) proteins encompass "variant" or "mutant" proteins, which terms are used interchangeably herein. Variant proteins differ from another (*i.e.*, parental) protein and/or from one another by a small number of amino acid residues. A variant may include one or more amino acid mutations (*e.g.*, amino acid deletion, insertion or substitution) as compared to the parental protein from which it is derived. Alternatively, or additionally, variants may have a specified degree of sequence identity with a reference protein or nucleic acid, *e.g.*, as determined using a sequence alignment tool, such as BLAST, ALIGN, and CLUSTAL. For example, variant proteins or nucleic acid may have at least about 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 99.5% amino acid sequence identity with a reference sequence and integer percentage therebetween

[0036] The term "wild-type" in reference to an amino acid sequence or nucleic acid sequence indicates that the amino acid sequence or nucleic acid sequence is a native or naturally-occurring sequence. As used herein, the term "naturally-occurring" refers to anything (*e.g.*, proteins, amino acids, or nucleic acid sequences) that is found in nature. Conversely, the term "non-naturally occurring" refers to anything that is not found in nature (*e.g.*, recombinant/engineered nucleic acids and protein sequences produced in the laboratory or modification of the wild-type sequence).

[0037] The term "derived from" encompasses the terms "originated from," "obtained from,"

“obtainable from,” “isolated from,” “purified from,” and “created from,” and generally indicates that one specified material finds its origin in another specified material or has features that can be described with reference to another specified material.

[0038] The terms “isolated,” “purified,” “separated,” and “recovered” as used herein refer to a material (e.g., a protein, nucleic acid, or cell) that is removed from at least one component with which it is naturally associated. For example, these terms may refer to a material which is substantially or essentially free from components which normally accompany it as found in its native state, such as, for example, an intact biological system. An isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

[0039] In one embodiment, there is disclosed a method for activating a zymogen of a transglutaminase, said method comprising contacting the zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.

[0040] While any Tgase can be used to practice the method disclosed herein, preferably, the transglutaminase is a microbial Tgase, and, more preferably, the microbial Tgase is a bacterial transglutaminase, such as a *Streptomyces* Tgase, and, most preferably, the bacterial Tgase is from *Streptomyces mobaraensis*, or a variant thereof. *Streptomyces mobaraensis* belongs to a large group of Gram-positive, filamentous soil bacteria with a complex life cycle.

[0041] Since the early 1990's, many microbial transglutaminase-producing strains have been found and production processes have been optimized and improved due to advances in bioprocess engineering and genetic engineering over the last three decades, as is well known to those skilled in the art.

[0042] Transglutaminase variants and methods of producing such variants are disclosed, for example, in PCT Publication Numbers WO 2016/170447, published on October 27, 2016, and WO 2019/094301, published on May 16, 2019.

[0043] Activating zymogen of a transglutaminase requires cleavage of a pro-sequence from a zymogen form of the enzyme, resulting in mature, catalytically active enzyme.

[0044] In the present disclosure, a zymogen of a Tgase is contacted with at least one immobilized protease to produce an activated form of a Tgase, and optionally, separating the activated Tgase from the immobilized protease.

[0045] Any protease that produces an activated form of a Tgase from the zymogen of a

Tgase may be used as described herein. Nonlimiting examples of proteases that may be immobilized for use in cleaving a pro-sequence from a Tgase as described herein include, but are not limited to, bacterial and fungal proteases. Preferably, the bacterial protease is a subtilisin. There can also be mentioned trypsin; proteinase K; proteinase T; Dispase® I; Dispase® II; Papain®; Bromelain®; Ficin®; Actinidine®; Protex 6L, Multifect® PR 6L; Protex® 7L, Multifect® PR 7L; Protex® 14L, Multifect® PR 14L; Protex® 15L, Multifect® PR 15L; Protex® 30L, Multifect® PR 30L; Protex® 40L, Optimase® PR 40L; Protex® 50FP, Multifect® PR 50G; Protex® 51FP, Multifect® PR 51G; Protex® 89L, Optimase® PR 89L, Alcalase®, Savinase®, Everlase®, Esperase®, Flavourzyme®, Neutrase®. Many immobilized proteases, such as Alcalase®, Savinase®, Everlase®, or Esperase®, are commercially available as a protease pre-immobilized and conjugated to a support matrix.

[0046] Conditions for contacting the zymogen of a Tgase with an immobilized protease include any conditions that are suitable for activity of a selected protease(s). Such conditions are readily ascertainable by one of skill in the art.

[0047] Activated zymogens of any of the Tgases described herein may be used in a variety of applications such as, but not limited to, food, pharmaceutical, cosmetic, healthcare, marine, paint, coating, energy (e.g., fracking fluid), plastic, packaging, and agricultural products. In some embodiments, the activated Tgase may be incorporated into HVAC systems, cooling ponds, water purification systems, or may be used in an industrial application, such as, but not limited to, pulp and paper processing.

[0048] The activated zymogens of Tgases may be used in healthcare products, personal care or cosmetic formulations, packaging (food, cosmetic, and pharmaceuticals), textile and leather production, paints and coatings, and marine applications including water treatment and purification. The activated zymogens of Tgases may be employed for permanently modifying proteins of interest, by way of example keratin and collagen, with dyes or proteins.

[0049] Preferably, the activated zymogen of any of the Tgases described herein is included in a personal care product, such as, but not limited to, bar soap, liquid soap (e.g., hand soap), hand sanitizer (including rinse off and leave-on alcohol based and aqueous-based hand disinfectants), preoperative skin disinfectant, cleansing wipes, disinfecting wipes, body wash, acne treatment products, antifungal diaper rash cream, antifungal skin cream, shampoo, conditioner, cosmetics (including but not limited to liquid or powder foundation, liquid or solid eyeliner, mascara, cream eye shadow, tinted powder, "pancake" type powder to be used dry or moistened, make up removal products, etc.), deodorant, antimicrobial creams, body lotion, hand cream, topical cream, aftershave lotion, skin toner,

mouth wash, toothpaste, sunscreen, and baby products such as, but not limited to, cleansing wipes, baby shampoo, baby soap, and diaper cream.

[0050] In some embodiments, an activated zymogen of a Tgase may be included in a wound care item, such as, but not limited to, wound healing ointments, creams, and lotions, wound coverings, burn wound cream, bandages, tape, and steri-strips, and medical articles such as medical gowns, caps, face masks, and shoe-covers, surgical drops, etc.

[0051] In other embodiments, the activated zymogen, of any of the Tgases described herein, may be included in an oral care product, such as mouth rinse, toothpaste, or dental floss coating, a veterinary or pet care product, a preservative composition, or a surface disinfectant, such as a disinfectant solution, spray or wipe.

[0052] In some embodiments, a product or composition which includes an activated zymogen of any of the Tgases as described herein, may further include one or more additional enzymes selected from acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1,4-glucanases, endo-beta-mannases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, beta-glucanases, tannases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, serine proteases, or combinations thereof.

[0053] In still some other embodiments, an activated zymogen of any of the Tgases described herein, may be included in a product to be used for long-lasting application of functional ingredients including UV-blocking sunscreens, and/or coloring agents, such as pigments or dyes. For example, it may be used in a composition for delivery of an active or functional ingredient to mammalian (e.g., human) skin, hair, or nails, such as, but not limited to, permanent (covalent) color modification of the surface of hair fibers. In some embodiments, such activated zymogens may be incorporated in a product to be applied topically and which bonds to the skin of an individual, such as a UV-blocking (sunscreen) product, or a cosmetic product. In some embodiments, the activated zymogen may be used to provide permanent application of color to the skin of an animal such as in leather processing. In some embodiments, the activated zymogen may be used to provide a permanent application of color in food processing.

[0054] Non-limiting embodiments of the foregoing disclosed herein include:

- [0055] 1. A method for activating a zymogen of a transglutaminase, said method comprising contacting the zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.
- [0056] 2. The method of embodiment 1 wherein the transglutaminase is a microbial transglutaminase.
- [0057] 3. The method of embodiment 2 wherein the microbial transglutaminase is *Streptomyces mobaraensis* transglutaminase or a variant thereof.
- [0058] 4. The method of any of embodiments 1, 2, or 3 wherein the at least one immobilized protease is selected from the group consisting of bacterial protease and fungal protease.
- [0059] 5. The method of embodiment 4 wherein the bacterial protease is subtilisin.
- [0060] 6. The method of any of embodiments 1, 2, 3, 4 or 5 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.
- [0061] 7. An activated transglutaminase produced by contacting a zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.
- [0062] 8. The activated transglutaminase of embodiment 9 wherein the transglutaminase is a microbial transglutaminase.
- [0063] 9. The activated transglutaminase of claim 10 wherein the microbial transglutaminase is *Streptomyces mobaraensis* transglutaminase or a variant thereof.
- [0064] 10. The activated transglutaminase of any of embodiments 7, 8, or 9 wherein the at least one immobilized protease is selected from the group consisting of bacterial protease and fungal protease.
- [0065] 11. The activated transglutaminase of embodiment 10 wherein the bacterial protease is subtilisin.
- [0066] 12. The activated transglutaminase of any of embodiments 7, 8, 9 10 or 11 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.

EXAMPLES

[0067] The following examples are intended to illustrate, but not limit, the invention. Accordingly, from the above discussion and the Examples, one skilled in the art can ascertain essential characteristics of this disclosure, and without departing from the spirit

and scope thereof, can make various changes and modifications to adapt to various uses and conditions.

[0068] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, *et al.*, *DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY*, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, *THE HARPER COLLINS DICTIONARY OF BIOLOGY*, Harper Perennial, N.Y. (1991) provide one of skill with a general dictionary of many of the terms used with this disclosure.

Example 1. Transglutaminase Activity Assays

[0069] Three different assays can be used to assess activity of an activated Tgase derived from a zymogen of Tgase and activated using at least one immobilized protease as described herein.

A) Colorimetric Activity Assay Using a Low Molecular Weight Substrate (≤ 1000 daltons)

[0070] Transglutaminase (Tgase) activity was measured herein using a colorimetric hydroxamate activity assay (Folk and Cole (1965) *J Biol Chemistry* 240(7):2951-2960). Briefly, the hydroxamate assay uses N-benzyloxycarbonyl-L-glutaminyl-glycine (ZQG) as a low molecular weight amine acceptor substrate and hydroxylamine as an amine donor. In the presence of transglutaminase, the hydroxylamine is incorporated to form Z-glutamylhydroxamate-glycine, which develops a colored complex with iron (III), detectable at 525 nm after incubation at 37 °C for 5-60 minutes. The calibration was performed using L-glutamic acid γ -monohydroxamate (Millipore Sigma) as standard. One unit of Tgase is defined as the amount of enzyme that catalyzes formation of 1 μ mol of the peptide derivative of γ -glutamylhydroxylamine per minute.

B) Casein Activity Assay Using a High Molecular Weight Substrate (>1000 daltons)

[0071] Transglutaminase (Tgase) activity is monitored by measuring the fluorescence (excitation wavelength 332 nm; emission wavelength 500 nm) using a BioTek Synergy H1 microplate reader. Transglutaminase-catalyzed covalent coupling (crosslinking) of monodansylcadaverine with N,N-dimethylcasein (high molecular weight substrate) produces a product that causes a shift in intensity and wavelength of fluorescence of the dansyl group now linked to the casein. The relative transglutaminase activity is shown by increase of fluorescence intensity over time.

C) HPLC Peptide Activity Assay Using a Low Molecular Weight Substrate (≤ 1000 daltons)

[0072] Transglutaminase-catalyzed crosslinking of N-benzyloxycarbonyl-L-glutaminyglycine (ZQG) (a low molecular weight substrate as described above) and lysine is monitored using RP-HPLC at 215 nm. ZQG and lysine are added at 1 g/L to a solution of 100 mM phosphate buffer at pH 7.0. Transglutaminase is added at 0.01-0.1 $\mu\text{g}/\mu\text{L}$ and allowed to react at 37°C, 300 RPM for 2 hours. The reaction is then quenched with 1:1 volume of 100% Acetonitrile. The samples are then run on an Agilent 1100 series HPLC through a Zorbax Eclipse Plus C18 column at 20-65% 0.08% TFA in Acetonitrile. Percent conversion of substrates to cross-linked product was calculated from the HPLC trace data to assess activity of the Tgase activated from a zymogen using at least one immobilized protease.

Example 2. Zymogen of Tgase (pro-Tgase) Expression in *Escherichia coli***A) Construction of expression plasmid for expression of a pro-Tgase**

[0073] The gene coding for the pro-Tgase was codon optimized for expression in *E. coli* based on the published amino acid sequence (Kanaji, et al. (1993) J. Biol. Chem. 268(16):11565-11572), synthesized with an additional C-terminal His tag, and cloned into a pET vector operatively linked to the T7 promoter under control of the lacI repressor. The expression vector also contains the pMB1 origin of replication and a kanamycin resistance gene. The resulting plasmid was transformed first into *E. coli* DH-10B, using standard methods known in the art. The transformants were isolated by subjecting the cells to kanamycin selection, as known in the art (See, e.g., US Pat. No. 8,383,346 and WO2010/144103, both of which are incorporated by reference herein, in their entirety), and the sequence of the pro-Tgase gene was verified by Sanger sequencing. The plasmid was recovered from a positive clone, using methods known in the art, and transformed into *E. coli* BL21(DE3) for expression.

B) Expression of recombinant pro-Tgase in *E. coli*

[0074] The *E. coli* strain BL21(DE3), containing the pro-Tgase expression vector, was cultured overnight in Luria broth at 37 °C until the culture reached saturation. The following morning, the culture was used to inoculate a shake flask containing a medium including glycerol, soy peptone, yeast extract, magnesium sulfate heptahydrate, and potassium phosphate monobasic, at 30-34 °C for up to 10 hours with continuous shaking. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1-1 mM and incubation was continued at 20-25 °C for up to 24 hours.

C) Protein harvest

[0075] Cells were harvested by centrifugation at 8000 x g for up to 60 minutes. The supernatant was discarded, and the pellet was resuspended to 20% w/v in 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 8. The cells were lysed using a high-pressure homogenizer at pressures from 15000-20000 psi. The crude lysates were clarified through centrifugation at 15000 x g for up to 60 minutes. The clarified lysate containing pro-Tgase was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by spectroscopy, and Tgase activity (as described in Example 1). Results depicted in *Figure 1* show that the zymogen of a Tgase, i.e., pro-Tgase, was expressed in *E.coli*.

[0076] Alternatively, the pro-Tgase may be secreted, for example, from a microbial strain that is known to those skilled in the art to secrete Tgase such as *Streptomyces mobaraensis* or *Bacillus subtilis*. The pellet is discarded, and the supernatant is recovered and assessed by SDS-PAGE, by spectroscopy, and activity (as described in Example 1 above).

Example 3. Immobilized Protease Screen for Activation of Tgase from zymogen of Tgase (pro-Tgase) in Clarified Lysate

[0077] Clarified lysate containing pro-Tgase, obtained as described in Example 2 above, was treated with a panel of commercially available immobilized proteases (Chiralvision Immozyme Protease Kit containing 20 immobilized proteases, Product Number: IMMPROT-2000). The protease treated clarified lysates were incubated with shaking at 30-45 °C, pH 5-8, for 0.1-24 hours. Protease loading was varied and optimized to deliver maximum activity within the fastest time. The protease-treated enzymes were compared on an SDS-PAGE gel, by spectroscopy, and by activity (as described in Example 1 above) for active enzyme. The products of activations described in Examples 3, 4, and 5 were analyzed using SDS-PAGE gels to visualize the ratio of Pro-Tgase to the active form of Tgase.

[0078] A 1-20 uL aliquot of the digest product was added to (Novex Bolt LDS Sample Buffer and Novex Bolt Sample Reducing Agent, mixed as directed), heated at 95°C for 5 minutes, then run on a 4-12% SDS-PAGE gel (Invitrogen NuPAGE 4-12% Bis-Tris Gel) for 30 minutes at 170 volts. Gels were stained with SimplyBlue™ Safe Stain (Invitrogen) for 30-60 minutes before being destained in deionized water and photographed.

[0079] Results are set forth in Table 1 and show relative activity of activated pro-Tgase (fold increase over activity of expressed zymogen, which may have some native activation during cell culture).

TABLE 1

Protease Source	Synonym	Manufacturer	Relative Activity (Fold Increase)
<i>Subtilisin from Bacillus sp.</i>	Alcalase®	Novozymes	10.3X
<i>Subtilisin from Bacillus sp.</i>	Savinase®	Novozymes	9.1X
<i>Subtilisin from Bacillus sp.</i>	Everlase®	Novozymes	11.3X
<i>Subtilisin from Bacillus sp.</i>	Esperase®	Novozymes	11.2X
<i>Bacillus licheniformis</i>	Protex® 6L, Multifect® PR 6L	Genencor	8.3X
<i>Bacillus amyloliquefaciens</i>	Protex® 7L, Multifect® PR 7L	Genencor	7.3X
<i>Thermolysin from Geobacillus sp.</i>	Protex® 14L, Multifect® PR 14L	Genencor	3.7X
<i>Trichoderma reesei</i>	Protex® 15L, Multifect® PR 15L	Genencor	1.3X
<i>Bacillus subtilis</i>	Protex® 30L, Multifect® PR 30L	Genencor	19.0X
<i>Bacillus subtilis</i>	Protex® 40L, Optimase® PR 40L	Genencor	16.6X
<i>Aspergillus oryzae</i> var.	Protex® 50 FP, Multifect® PR 50G	Genencor	2.9X
<i>Aspergillus oryzae</i>	Protex® 51 FP, Multifect® PR 51G	Genencor	5.6X
<i>Bacillus subtilis</i>	Protex® 89L, Optimase® PR 89L	Genencor	4.4X
<i>Aspergillus niger</i>	Acid protease	Aum Enzymes	2.7X
<i>Bacillus subtilis</i>	Neutral protease	Aum Enzymes	3.8X
<i>Bacillus subtilis</i>	Alkaline protease	Aum Enzymes	9.8X
<i>Carica papaya</i>	Papain®	Sigma-Aldrich	1.2X
pineapple stem	Bromelain®	Sigma-Aldrich	1.8X
fig tree latex	Ficin®	Sigma-Aldrich	2.8X
kiwi	Actinidine®	KiwiEnzyme	1.2X

Example 4. Immobilized Protease pH Screen for Activation of Zymogen of the Tgase (pro-Tgase) in Clarified Lysate

[0080] Clarified lysate containing pro-Tgase, as described in Example 2, was treated with either immobilized Protex® 30L (Multifect® PR 30L); or immobilized Protex® 40L (Optimase® PR 40L) (Chiralvision; Product Numbers: IMMP30-COV-2, IMMP40-COV-2, respectively). The protease-treated clarified lysates were incubated with shaking at 30 or 45 °C, pH 5-9 for 0.1-24 hours. Protease loading was varied and optimized to deliver maximum activity within the fastest time. The protease treated enzymes were compared on an SDS-PAGE gel, by spectroscopy, and by activity (as described in Example 1) for active enzyme. Results are shown in Table 2. These results show relative activity of Tgase activated from zymogen (i.e., activated pro-Tgase) at 30 °C (increase over activity of expressed zymogen, which may have some native activation during cell culture), as described in Example 3 above.

TABLE 2

	pH								
	5	5.5	6	6.5	7	7.5	8	8.5	9
IMMP30-COV-2	+	+	+	+	+	++	++	++	++
IMMP40-COV-2	++	++	++	++	++	++	++	++	++

+ indicates an improvement of 10 to 15-fold over pro-Tgase

++ indicates an improvement greater than 15-fold over pro-Tgase

Example 5. Substrate Specificity of Activated Pro-Tgase Using Immobilized Protease

[0081] Clarified lysate containing Pro-Tgase was prepared as described in Example 2. The lysate was diluted to a final concentration of 0.5-3 g/L Pro-Tgase at pH 5-8 using bis-tris propane, phosphate, tris-acetate, or similar buffer. A sample of 5-30 mg of immobilized protease was added to a 0.75 mL glass vial. Then, 0.4-0.6 mL of the buffered the lysate was added. A total of 22 immobilized proteases (Chiralvision Immozyme Protease Kit containing 22 immobilized proteases, Product Number: IMMPROT-2200) were tested. The mixture was incubated at 30-45°C, while mixing at 200-400 rpm. Product was sampled at time points from 0.1-24 hours for further testing. The progress of the reaction was monitored using assays described in Examples 1B (high molecular weight) and 1C (small molecular weight) to determine when maximum activity was reached for each protease. Table 3 shows percent conversions calculated from HPLC assay results (as described in Example 1C) above for 5 of the 22 protease activations. Furthermore, Table 3 also shows the ease with which the reaction can be quenched when maximum activity of the Tgase is achieved. Then the activated Tgase can be separated from the immobilized protease by filtration and carried forward in reactions with either high molecular weight substrates (>1000 daltons) or low molecular weight substrates (≤1000 daltons) as shown in Table 4, or isolated for protein sequencing to determine cleavage sites. The results presented in Table 4 indicate that once the Tgase is activate it will react with both high and low molecular weight substrates although the degree to which the Tgase reacts with low or high molecular weight substrate may vary slightly.

TABLE 3 – HPLC Activity Assay Results (low molecular weight substrate)

	0 hr	1 hr	2 hr	3 hr	6 hr
Savinase®	1.1	42.0	68.5	45.0	34.1
Flavourzyme®	1.1	29.0	33.3	39.1	34.5
Everlase®	1.1	37.1	40.7	44.0	33.8
Protex 7L®	1.1	13.1	23.0	29.6	33.9
Protex 14L®	1.1	3.8	8.1	18.6	28.4

TABLE 4 – Reactivity of Activated Tgase with High and Low MW substrates

Enzyme Name	Chiralvision Product number	Protease Source	Enzyme manufacturer	Activity on substrates <1000 Da	Activity on substrates >1000 Da
Alcalase®	IMMALC-COV-2	<i>Subtilisin from Bacillus sp.</i> (bacterial)	Novozymes	+++	+++
Savinase®	IMMSAV-COV-2	<i>Subtilisin from Bacillus sp.</i> (bacterial)	Novozymes	+++	+++
Everlase®	IMMEVE-COV-2	<i>Subtilisin from Bacillus sp.</i> (bacterial)	Novozymes	+++	+++
Esperase®	IMMESP-COV-2	<i>Subtilisin from Bacillus sp.</i> (bacterial)	Novozymes	+++	+++
Protex® 6L, Multifect® PR 6L	IMMP6-COV-2	<i>Bacillus licheniformis</i> (bacterial)	Genencor	+++	+++
Protex® 7L, Multifect® PR 7L	IMMP7-COV-2	<i>Bacillus amyloliquefaciens</i> (bacterial)	Genencor	++	+++
Protex® 14L, Multifect® PR 14L	IMMP14-COV-2	<i>Thermolysin from Geobacillus sp.</i> (bacterial)	Genencor	++	+++
Protex® 15L, Multifect® PR 15L	IMMP15-COV-2	<i>Trichoderma reesei</i> (fungal)	Genencor	-	-
Protex® 30L, Multifect® PR 30L	IMMP30-COV-2	<i>Bacillus subtilis</i> (bacterial)	Genencor	++	+++
Protex® 40L, Optimase® PR 40L	IMMP40-COV-2	<i>Bacillus subtilis</i> (bacterial)	Genencor	+++	+++
Protex® 50 FP, Multifect® PR 50G	IMMP50-COV-2	<i>Aspergillus oryzae</i> var. (fungal)	Genencor	-	-

Protex® 51 FP, Multifect® PR 51G	IMMP51-COV-2	<i>Aspergillus oryzae</i> (fungal)	Genencor	+	+
Protex® 89L, Optimase® PR 89L	IMMP89-COV-2	<i>Bacillus subtilis</i> (bacterial)	Genencor	+++	++
Acid protease	IMMAUAC-COV-2	<i>Aspergillus niger</i> (fungal)	Aum Enzymes	-	-
Neutral protease	IMMAUNE-COV-2	<i>Bacillus subtilis</i> (bacterial)	Aum Enzymes	-	-
Alkaline protease	IMMAUAL-COV-2	<i>Bacillus subtilis</i> (bacterial)	Aum Enzymes	+++	+++
Papain®	IMMPAP-COV-2	<i>Carica papaya</i> (plant)	Sigma-Aldrich	+	-
Bromelain®	IMMBRO-COV-2	pineapple stem (plant)	Sigma-Aldrich	-	-
Ficin®	IMMFIC-COV-2	fig tree latex (plant)	Sigma-Aldrich	-	-
Actinidine	IMMACT-COV-2	kiwi (plant)	KiwiEnzyme	-	+
Flavourzyme® 100L	IMMFLAV-COV-2	<i>Aspergillus oryzae</i> (fungal)	Novozymes	++	+++
Neutrase® 0.8 L	IMMNEU-COV-2	<i>Bacillus amyloliquefaciens</i> (bacterial)	Novozymes	+	-

- indicates less than 2-fold improvement over pro-Tgase

+ indicates between 2 and 20-fold increase in activity over pro-Tgase

++ indicates between 20 and 30-fold increase in activity over pro-Tgase

+++ indicates greater than 30-fold increase in activity over pro-Tgase

CLAIMS

What is claimed is:

1. A method for activating a zymogen of a transglutaminase, said method comprising contacting the zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.
2. The method of claim 1 wherein the transglutaminase is a microbial transglutaminase.
3. The method of claim 2 wherein the microbial transglutaminase is *Streptomyces mobaraensis* transglutaminase or a variant thereof.
4. The method of claim 1, 2, or 3 wherein the at least one immobilized protease is selected from the group consisting of bacterial protease and fungal protease.
5. The method of claim 4 wherein the bacterial protease is subtilisin.
6. The method of claim 1, 2, or 3 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.
7. The method of claim 4 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.
8. The method of claim 5 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.
9. An activated transglutaminase produced by contacting a zymogen with at least one immobilized protease to produce an active form of transglutaminase and, optionally, separating the activated transglutaminase from the immobilized protease.
10. The activated transglutaminase of claim 9 wherein the transglutaminase is a microbial transglutaminase.
11. The activated transglutaminase of claim 10 wherein the microbial transglutaminase is *Streptomyces mobaraensis* transglutaminase or a variant thereof.
12. The activated transglutaminase of claim 9, 10 or 11 wherein the at least one immobilized protease is selected from the group consisting of bacterial protease and fungal protease.
13. The activated transglutaminase of claim 12 wherein the bacterial protease is subtilisin.
14. The activated transglutaminase of claim 9, 10 or 11 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.
15. The activated transglutaminase of claim 12 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.

16. The activated transglutaminase of claim 13 wherein the immobilized protease is immobilized on a support suitable for producing an activated transglutaminase.

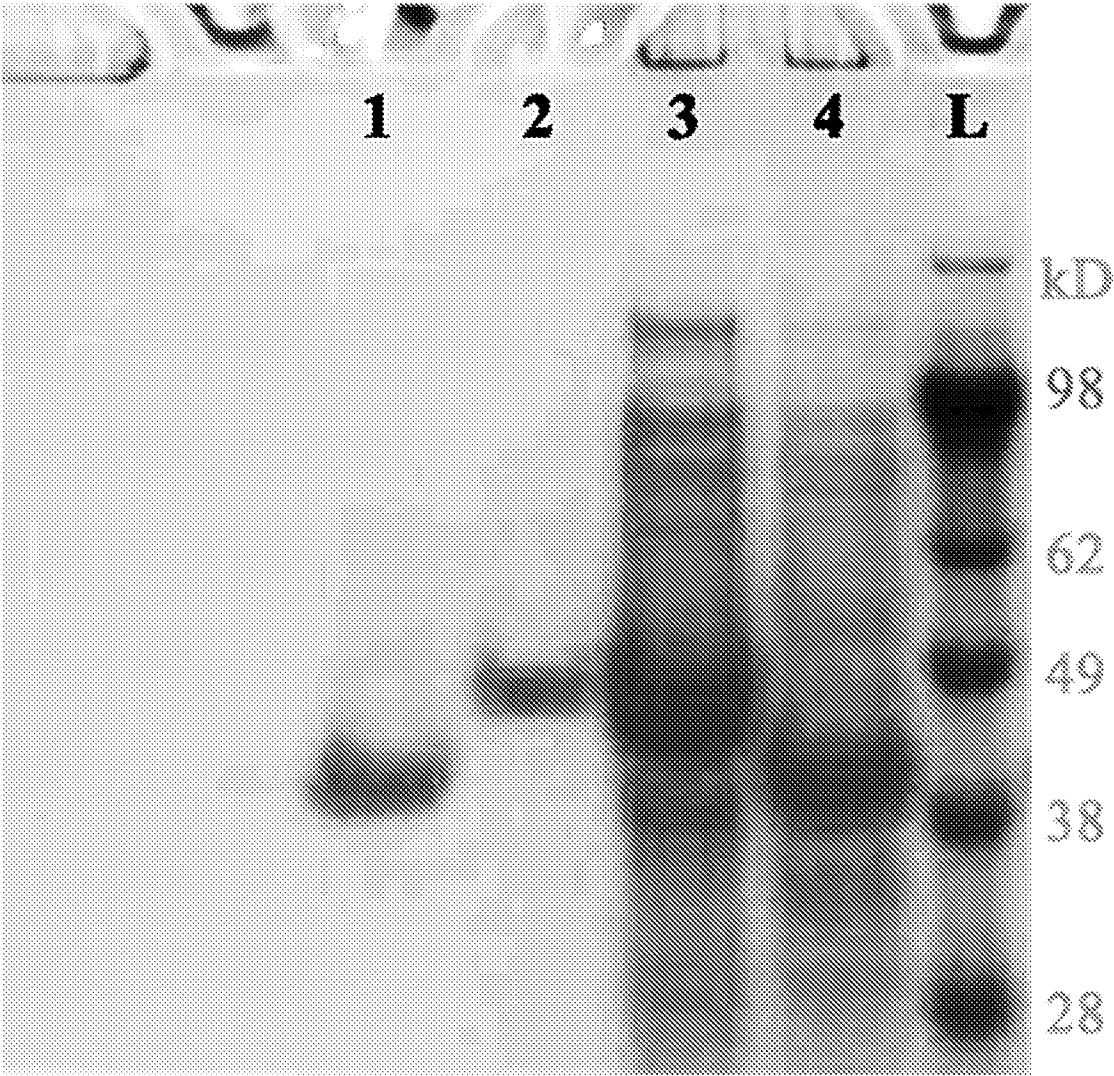


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/019738

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/10 C12N9/52 C12N9/58 C12N11/00 C12P21/06 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N C12R C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/15234 A1 (NOVO NORDISK AS [DK])	1, 4, 6-9,
Y	5 August 1993 (1993-08-05)	12, 14-16
	claim 20	2, 3, 5,
	-----	10, 11, 13
	-/--	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 17 June 2022		Date of mailing of the international search report 11/07/2022
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Griesinger, Irina

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2022/019738

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Y	WO 2011/133704 A2 (WHITEHEAD BIOMEDICAL INST [US]; GUIMARAES CARLA [US] ET AL.) 27 October 2011 (2011-10-27) claims 27-29 -----	1-16
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Y	PASTERNAK ET AL: "Bacterial pro-transglutaminase from Streptovercillium mobarensense", EUROPEAN JOURNAL OF BIOCHEMISTRY, PUBLISHED BY SPRINGER-VERLAG ON BEHALF OF THE FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES, vol. 257, 1 January 1998 (1998-01-01), pages 570-576, XP002127089, ISSN: 0014-2956, DOI: 10.1046/J.1432-1327.1998.2570570.X abstract -----	1-16
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International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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