

EXPRESSION OF MODIFIED PROTEINS IN A PEROXISOME

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 62/847,769, filed May 14, 2019, which is hereby incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING AND TABLES IN ELECTRONIC FORMAT

[0002] This application is filed with an electronic sequence listing entitled PBFAB001WO2SEQLIST.TXT, created on May 7, 2020 which is 235 KB in size. The information in the electronic sequence listing is hereby incorporated by reference in its entirety.

FIELD

[0003] Methods and compositions are provided herein for genetically modifying cells to produce proteins and protein precursors that for example may be used in artificial materials.

BACKGROUND

[0004] There is a need in the art for improved methods of producing and modifying proteins in cells. Proteins produced and modified in cells find use in a variety of ways.

SUMMARY

[0004a] In one aspect, provided herein is a method of producing a modified protein in a peroxisome, the method comprising: providing a cell comprising the peroxisome; introducing a first nucleic acid into the cell, wherein the first nucleic acid comprises a first sequence encoding a heterologous protein fused to a peroxisome-targeting sequence; introducing a second nucleic acid into the cell, wherein the second nucleic acid comprises a second sequence encoding a heterologous modification enzyme fused to a peroxisome-targeting sequence; and culturing the cell under conditions such that the heterologous protein

and the heterologous modification enzyme are expressed in the cell, wherein the heterologous modification enzyme modifies the heterologous protein in the peroxisome to produce the modified protein.

[0004b] In another aspect, provided herein is a eukaryotic cell for producing a modified protein in a peroxisome, manufactured by a method as described herein.

[0004c] In another aspect, provided herein is a eukaryotic cell for producing a modified protein in a peroxisome, the cell comprising: the peroxisome; a first nucleic acid comprising a sequence encoding a heterologous protein fused to a peroxisome-targeting sequence; and a second nucleic acid encoding a heterologous modification enzyme fused to a peroxisome-targeting sequence, wherein the heterologous modification enzyme is configured to modify the heterologous protein to produce a modified protein.

[0004d] In another aspect, provided herein is a eukaryotic cell for producing a modified protein in a peroxisome, wherein the eukaryotic cell is capable of expressing: a heterologous protein fused to a peroxisome-targeting sequence, and a heterologous modification enzyme fused to a peroxisome-targeting sequence, wherein the heterologous modification enzyme is configured to modify the heterologous protein to produce the modified protein.

[0004e] In another aspect, provided herein is a method of producing a modified protein in a eukaryotic cell, wherein the eukaryotic cell expresses a heterologous modification enzyme fused to a peroxisome-targeting sequence, comprising: providing a cell manufactured by the method as described herein, or a cell as described herein; expressing a heterologous protein in the eukaryotic cell, wherein the heterologous protein is fused to a peroxisome-targeting sequence; and culturing the eukaryotic cell under conditions such that the heterologous modification enzyme modifies the heterologous protein in the peroxisome to produce a modified protein.

[0004f] In another aspect, provided herein is a method of producing a modified protein in a eukaryotic cell, wherein the cell comprises a peroxisome, wherein the eukaryotic cell expresses a heterologous modification enzyme fused to a peroxisome-targeting sequence, comprising: expressing a heterologous protein in a eukaryotic cell, wherein the heterologous protein is fused to a peroxisome-targeting sequence; and culturing the eukaryotic cell under

conditions such that the heterologous modification enzyme modifies the heterologous protein in a peroxisome to produce a modified protein.

[0004g] In another aspect, provided herein is a method of producing a modified protein, comprising: culturing a eukaryotic cell containing a peroxisome under conditions such that the modified protein is produced, wherein the eukaryotic cell expresses: a heterologous protein fused to a peroxisome-targeting sequence, and a heterologous modification enzyme fused to a peroxisome-targeting sequence; wherein the heterologous modification enzyme modifies the heterologous protein to produce the modified protein in the peroxisome under the culture conditions.

[0004h] In another aspect, provided herein is a method of increasing yield of a modified protein, comprising: culturing a eukaryotic cell containing a peroxisome under conditions such that the modified protein is produced, wherein the eukaryotic cell expresses: a heterologous protein fused to a peroxisome-targeting sequence, wherein expression of the heterologous protein is under the influence of a promoter, and a heterologous modification enzyme fused to a peroxisome-targeting sequence; wherein the heterologous modification enzyme modifies the heterologous protein to produce the modified protein in the peroxisome under the culture conditions.

[0004i] In another aspect, provided herein is a kit for producing a modified protein in a peroxisome in a cell, comprising: a first nucleic acid construct comprising GFP-x-ePTS1 or x-FLAG-ePTS1, wherein x is a nucleic acid sequence encoding a heterologous protein to be targeted to a peroxisome; and a second nucleic acid construct comprising GFP-y-ePTS1 or y-FLAG-ePTS1, wherein y is a nucleic acid sequence encoding a modification enzyme to be targeted to the peroxisome, wherein the modification enzyme is configured to modify the heterologous protein in the peroxisome.

[0005] Described herein are methods for producing proteins that can act as precursors for materials, such as substrates for products in film development; capsules for pills (gelatin in drug and nutraceuticals); food additives (e.g. all things gelatin) and collagen for food stuffs and synthetic meats, textiles such as synthetic leather, beauty products, and biomedical materials (scaffolds, sutures, grafts, expanding cells, gels, etc.) are contemplated. The use of such methods may also provide materials that would reduce the product carbon footprint from standard manufacturing methods that are used today.

WHAT IS CLAIMED IS:

1. A method of producing a modified protein in a peroxisome, the method comprising:
 - providing a cell comprising the peroxisome;
 - introducing a first nucleic acid into the cell, wherein the first nucleic acid comprises a first sequence encoding a heterologous protein fused to a peroxisome-targeting sequence;
 - introducing a second nucleic acid into the cell, wherein the second nucleic acid comprises a second sequence encoding a heterologous modification enzyme fused to a peroxisome-targeting sequence; and
 - culturing the cell under conditions such that the heterologous protein and the heterologous modification enzyme are expressed in the cell,
 - wherein the heterologous modification enzyme modifies the heterologous protein in the peroxisome to produce the modified protein.
2. The method of claim 0, wherein the cell is a eukaryotic cell.
3. The method of any one of claims 0-2, wherein the cell is a yeast cell.
4. The method of any one of claims 0-3, wherein the cell is selected from *Arxula*, *Candida*, *Hansenula*, *Kluyveromyces*, *Komagataella*, *Ogataea*, *Pichia*, *Saccharomyces*, or *Yarrowia*.
5. The method of any one of claims 0-4, wherein the first and/or second nucleic acid comprises a promoter(s).
6. The method of claim 5, wherein the promoter is constitutive or inducible.
7. The method of any one of claims 0-6, wherein the peroxisome-targeting sequence comprises a sequence set forth in SEQ ID NO: 1 (SLK), SEQ ID NO: 2 (RLXXXXX(H/Q)L), or SEQ ID NO: 3 (LGRGRRSKL).
8. The method of any one of claims 0-7, wherein the protein comprises a tag.
9. The method of claim 8, wherein the tag is cleavable.
10. The method of any one of claims 0-9, wherein the method further comprises introducing a third nucleic acid into the cell, wherein the third nucleic acid comprises a third sequence encoding a second heterologous modification enzyme fused to a peroxisome-targeting sequence.

11. The method of any one of claims 0-10, wherein the heterologous protein has a molecular weight of 1 Da, 5 Da, 10 Da, 20 Da, 30 Da, 40 Da, 50 Da, 60 Da, 70 Da, 80 Da, 90 Da, 100 Da, 200 Da, 300 Da, 400 Da, 500 Da, 600 Da, 700 Da, 800 Da, 900 Da, 1 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa, 40 kDa, 50 kDa, 60 kDa, 70 kDa, 80 kDa, 90 kDa, 100 kDa, 110 kDa, 120 kDa, 130 kDa, 140 kDa, 150 kDa, 160 kDa, 170 kDa, 180 kDa, 190 kDa, 200 kDa, 210 kDa, 220 kDa, 230 kDa, 240 kDa, 250 kDa, 260 kDa, 270 kDa, 280 kDa, 290 kDa, or 300 kDa, or any size in between a range defined by any two aforementioned values, or up to 300 kDa.

12. The method of any one of claims 0-11, wherein the enzyme creates a modification.

13. The method of any of claim 12, wherein the modification is hydroxylation, protein folding, oxidation, proteolysis, phosphorylation, dephosphorylation, and/or isomerization.

14. The method of any one of claims 0-13, wherein the enzyme comprises prolyl hydroxylases, lysyl oxidases, a protein chaperone or prolyl isomerase.

15. The method of any one of claims 0-14, wherein the enzyme is selected from a prolyl isomerase, a protein disulfide isomerase, a hydroxyl transferase, or a prolyl hydroxylase.

16. The method of any one of claims 0-15, wherein the protein comprises collagen, gelatin or silk protein.

17. The method of any one of claims 0-16, wherein the nucleic acid is codon optimized for protein expression in a eukaryotic cell, such as a yeast cell.

18. The method of any one of claims 0-17, wherein the enzyme comprises prolyl hydroxylase or prolyl isomerase.

19. The method of any one of claims 0-18, wherein the protein is collagen, the collagen is modified resulting in a Type I heterotrimer, Type 1 alpha homotrimer, or Type III homotrimer collagen.

20. The method of any one of claims 0-19, wherein the heterologous protein comprises Col1A1 or Col1A2.

21. The method of any one of claims 0-20, wherein the enzyme comprises prolyl-4-hydroxylase.

22. The method of claim 21, wherein the prolyl-4-hydroxylase is genetically modified to have a deletion of a PDI domain.

23. The method of any one of claims 0-22, wherein the enzymes are genetically modified for improved expression and import into the peroxisome.

24. The method of any one of claims 0-23, wherein the proteins are genetically modified for improved expression and import into the peroxisome.

25. The method of any of claims 0-24, wherein fusion of the heterologous protein to the peroxisome targeting sequence results in targeting of the heterologous protein to the peroxisome, thereby separating the heterologous protein from an enzyme not targeted to the peroxisome.

26. The method of any of claims 0-25 wherein fusion of the modification enzyme to the peroxisome targeting sequence results in targeting of the modification enzyme to the peroxisome, thereby separating the modification enzyme from a substrate or enzyme not targeted to the peroxisome.

27. The method of any one of claims 0-26, wherein the heterologous protein comprises COLsyn2, COLsyn3, or an amino acid sequence at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the amino acid sequence of COLsyn2 or COLsyn3.

28. The method of any one of claims 0-27, wherein the first nucleic acid is engineered to replace at least one hydrophobic amino acid with a hydrophilic or non-hydrophobic amino acids in the heterologous protein as compared to an unmodified or naturally occurring first nucleic acid.

29. A eukaryotic cell for producing a modified protein in a peroxisome, manufactured by a method of any one of claims 0-28.

30. A eukaryotic cell for producing a modified protein in a peroxisome, the cell comprising:

the peroxisome;

a first nucleic acid comprising a sequence encoding a heterologous protein fused to a peroxisome-targeting sequence; and

a second nucleic acid encoding a heterologous modification enzyme fused to a peroxisome-targeting sequence,

wherein the heterologous modification enzyme is configured to modify the heterologous protein to produce a modified protein.

31. A eukaryotic cell for producing a modified protein in a peroxisome, wherein the eukaryotic cell is capable of expressing:

a heterologous protein fused to a peroxisome-targeting sequence, and
a heterologous modification enzyme fused to a peroxisome-targeting sequence,
wherein the heterologous modification enzyme is configured to modify the heterologous protein to produce the modified protein.

32. The eukaryotic cell of any one of claims 30-31, wherein the protein is modified in the peroxisome.

33. The eukaryotic cell of any one of claims 29-32, wherein the cell is *Pastoris*.

34. The eukaryotic cell of any one of claims 29-33, wherein the peroxisome-targeting sequence comprises a sequence set forth in SEQ ID NOs: 1, 2, or 3.

35. The eukaryotic cell of any one of claims 29-34, wherein the cell further comprises a third nucleic acid encoding a protein fused to a peroxisome-targeting sequence.

36. A method of producing a modified protein in a eukaryotic cell, wherein the eukaryotic cell expresses a heterologous modification enzyme fused to a peroxisome-targeting sequence, comprising:

providing a cell manufactured by the method of any one of claims 0-28, or a cell of any one of claims 29-35;

expressing a heterologous protein in the eukaryotic cell, wherein the heterologous protein is fused to a peroxisome-targeting sequence; and

culturing the eukaryotic cell under conditions such that the heterologous modification enzyme modifies the heterologous protein in the peroxisome to produce a modified protein.

37. A method of producing a modified protein in a eukaryotic cell, wherein the cell comprises a peroxisome, wherein the eukaryotic cell expresses a heterologous modification enzyme fused to a peroxisome-targeting sequence, comprising:

expressing a heterologous protein in a eukaryotic cell, wherein the heterologous protein is fused to a peroxisome-targeting sequence; and

culturing the eukaryotic cell under conditions such that the heterologous modification enzyme modifies the heterologous protein in a peroxisome to produce a modified protein.

38. A method of producing a modified protein, comprising:

culturing a eukaryotic cell containing a peroxisome under conditions such that the modified protein is produced, wherein the eukaryotic cell expresses:

a heterologous protein fused to a peroxisome-targeting sequence, and

a heterologous modification enzyme fused to a peroxisome-targeting sequence;

wherein the heterologous modification enzyme modifies the heterologous protein to produce the modified protein in the peroxisome under the culture conditions.

39. A method of increasing yield of a modified protein, comprising:

culturing a eukaryotic cell containing a peroxisome under conditions such that the modified protein is produced, wherein the eukaryotic cell expresses:

a heterologous protein fused to a peroxisome-targeting sequence, wherein expression of the heterologous protein is under the influence of a promoter, and

a heterologous modification enzyme fused to a peroxisome-targeting sequence; wherein the heterologous modification enzyme modifies the heterologous protein to produce the modified protein in the peroxisome under the culture conditions.

40. The method of claim 39, wherein production of the heterologous protein is induced by a chemical inducer.

41. The method of claim 39 or 40, wherein the method further comprises increasing cargo of the peroxisome, wherein increasing cargo of the peroxisome is performed by providing oleic acid or methanol to the eukaryotic cell.

42. A kit for producing a modified protein in a peroxisome in a cell, comprising:

a first nucleic acid construct comprising GFP-x-ePTS1 or x-FLAG-ePTS1, wherein x is a nucleic acid sequence encoding a heterologous protein to be targeted to a peroxisome; and

a second nucleic acid construct comprising GFP-y-ePTS1 or y-FLAG-ePTS1, wherein y is a nucleic acid sequence encoding a modification enzyme to be targeted to the peroxisome, wherein the modification enzyme is configured to modify the heterologous protein in the peroxisome.