METHOD FOR PRODUCTION OF RECOMBINANT *E. COLI* ASPARAGINASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 62/578,302, filed October 27, 2017, which is incorporated herein by reference.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 16, 2018, is named 38194-751_601_SL.txt and is 54,846 bytes in size.

BACKGROUND OF THE INVENTION

[0003] L-asparaginase catalyses the conversion of L-asparagine to L-aspartate. L-asparaginase type II from the bacterium *E. coli* is a tetrameric high-affinity periplasmic enzyme produced with a cleavable secretion signal sequence. Also known as L-asparagine amidohydrolase, it is the active ingredient in commercially approved drug products indicated for the treatment of patients having acute lymphoblastic leukemia (ALL). For example, Elspar®, approved in the United States for ALL treatment, has as its active ingredient *E. coli* L-asparaginase type II. Oncaspar® (pegaspargase) contains L-asparaginase (L-asparagine amidohydrolase) that is covalently conjugated to monomethoxypolyethylene glycol (mPEG). Oncaspar is approved in the United States for treatment of first line ALL, as well as ALL and hypersensitivity to native *E. coli* asparaginase. *E. coli* L-asparaginase type II is also used to treat other neoplastic conditions. The *E. coli* asparaginase can be purified from a culture of *E. coli* to yield the drug substance, e.g., genetically modified *E. coli* that is deficient in native asparaginase. In some cases, it is expressed from a gene fusion with a heterologous secretion signal peptide.

[0004] Periplasmic localization of asparaginase can provide advantages in different expression systems including reduced production of inclusion bodies, reduced proteolysis and generation of an authentic protein N-terminus. Expression yields can be lower due to the limited availability of secretion pathway cofactors and/or the spatial restriction of the periplasmic space. Cytoplasmic expression of recombinant asaparaginase, can generate higher yields if the bacterial host cell cytoplasmic environment presents few penalties in regards to solubility, degradation and mis-folding of the asparaginase monomer.

SUMMARY OF THE INVENTION

[0004a] In one aspect, provided herein is a method for producing a recombinant type II asparaginase, the method comprising: culturing a *Pseudomonadales* host cell deficient in the expression of at least one native asparaginase in a culture medium and expressing the recombinant type II asparaginase in the periplasm of the *Pseudomonadales* host cell from an expression construct comprising a nucleic acid encoding the recombinant type II asparaginase; wherein the recombinant type II asparaginase encoded by the nucleic acid comprises an amino acid sequence at least 85% identical to SEQ ID NO: 1, wherein the

recombinant type II asparaginase is produced in the periplasm at a yield of about 33% to about 65% total cell protein (TCP) in soluble form.

[0004b] In one aspect, provided herein is a use of the recombinant type II asparaginase produced by the method as described herein in the manufacture of a medicament for the treatment of patients with neoplastic conditions.

[0004c] In one aspect, provided herein is the recombinant type II asparaginase produced by the method as described herein for use in therapy.

[0005] Provided herein are methods for producing a recombinant type II asparaginase. In some examples, the method comprises: culturing a *Pseudomonadales* host cell in a culture medium and expressing the recombinant asparaginase in the periplasm of the *Pseudomonadales* host cell from an expression construct comprising a nucleic acid encoding the recombinant asparaginase; wherein the recombinant asparaginase is produced in the periplasm at a yield of about 31% to about 60% TCP soluble asparaginase. In some examples, the soluble recombinant asparaginase is produced in the periplasm at a yield of about 10 g/L to about 38 g/L. In some examples, the method further comprises measuring the activity of an amount of the recombinant type II asparaginase produced, using an activity assay. In some examples, the nucleic acid encoding the recombinant asparaginase is optimized for expression in the host cell. In some examples, the asparaginase is an *Escherichia coli* L-asparaginase type II. In some examples, the nucleic acid encoding the recombinant asparaginase comprises a sequence at least 85% homologous to SEQ ID NO: 1 or 3. In some examples, the recombinant asparaginase has an amino acid sequence at least 85% homologous to SEQ ID NO: 2. In some examples, expression of the recombinant asparaginase is induced with addition of IPTG to the culture media. In some examples, the IPTG is at a concentration in the culture media of about 0.14 mM to about 0.3 mM. In some examples, expression of the recombinant asparaginase is induced when the *Pseudomonad* host cell has grown to a wet cell weight of about 0.05 g/g to about 0.4 g/g. In some examples, the *Pseudomonadales* host cell is cultured at a pH of about 5.0 to about 8.0. In some examples, the *Pseudomonadales* host cell is cultured at a temperature of about 22 °C to about 33 °C. In some examples, the *Pseudomonadales* host cell is cultured in a media comprising about 3g/L to about 8 g/L mannitol. In examples, the *Pseudomonadales* host cell is cultured in a media comprising no mannitol. In some examples, the *Pseudomonadales* host cell is cultured in a media comprising about 0.1 mM to about 1 mM $CaCl_2$. In some examples, the *Pseudomonadales* host cell is a *Pseudomonas fluorescens* cell. In some examples, the *Pseudomonadales* host cell is deficient in the expression of one or more asparaginases. In some examples, the *Pseudomonadales* host cell is deficient in the expression of one or more native asparaginases. In some examples, the deficiently expressed native asparaginase is a type I asparaginase. In some examples, the deficiently expressed native asparaginase is a type II asparaginase. In some examples, the *Pseudomonadales* host cell is deficient in the expression of one or more proteases. In some examples, the *Pseudomonadales* host cell overexpresses one or more folding modulators. In some examples, the *Pseudomonadales* host cell is selected from at least one of: a host cell that overexpresses LepB; a host cell that overexpresses Tig; a

host cell that overexpresses DsbA, DsbC, and Skp (DsbAC-Skp); a host cell that is deficient in Lon, HslUV, DegP1, DegP2, Prc, AprA, DegP2 S219A, Prc1, or AprA; a host cell that is deficient in AspG1; a host cell that is deficient in AspG2; a host cell that does not overexpress a folding modulator, and is not deficient in a protease; a host cell that does not overexpress a folding modulator, is not deficient in a protease; and is not deficient in AspG1; a host cell that does not overexpress a folding modulator, is not deficient in a protease; and is not deficient in AspG2; and a host cell that does not overexpress a folding modulator, is not deficient in a protease; and is not deficient in AspG1 or AspG2. In some examples, the *Pseudomonadales* host cell is selected from: a host cell that is deficient in Lon and HslUV; a host cell that is deficient in Lon, DegP1, DegP2, Prc, and AprA; a host cell that is deficient in Lon, DegP1, DegP2 S219A, Prc1, and AprA, and overexpresses DsbAC-Skp; a host cell that is deficient in AspG1 and/or AspG2; a host cell that is deficient in AspG1 and/or AspG2, and overexpresses Tig; a host cell that is deficient in AspG1 and/or AspG2, and overexpresses LepB; a host cell that is deficient in AspG1 and/or AspG2, and deficient in Lon and HslUV; a host cell that is deficient in AspG1 and/or AspG2, and deficient in Lon, DegP1, DegP2, Prc, and AprA; and a host cell that is deficient in AspG1 and/or AspG2, Lon, DegP1, DegP2, Prc1, and AprA, and overexpresses DsbAC-Skp. In some examples, the expression construct comprises a secretion leader. In some examples, the secretion leader is selected from the group comprising the *Pseudmonadales* secretion leaders AnsB, 8484, IBP-S31A, pbp, 8584, LAO, Azu, PbpA20V, CupC2, and the *Escherichia coli* K-12 AnsB secretion leader. In some examples, the secretion leader directs transfer of the recombinant asparaginase produced to the periplasm of the *Pseudomonadales* host cell. In some examples, the method further comprises comparing the measured activity of the recombinant type II asparaginase produced with an activity measured in the same amount of a control type II asparaginase using the same activity assay. In some examples, the control type II asparaginase comprises an *E. coli* type II asparaginase that has been commercially approved for use in patients. In some examples, the recombinant type II asparaginase produced is selected for use in patients when it has about 80% to about 120% of the activity of the control type II asparaginase. In some examples, the recombinant type II asparaginase produced is modified to increase half-life in patients. In some examples, the recombinant type II asparaginase expressed from the expression construct is a recombinant *E. coli* type II asparaginase, wherein the nucleic acid encodes the recombinant *E. coli* type II asparaginase operably linked to the *P. fluorescens* AnsB secretion leader, and wherein the recombinant *E. coli* type II asparaginase is produced in the periplasm at a yield that is about 20% to about 100% greater than that of a recombinant *P. fluorescens* type II asparaginase produced in the periplasm by the same method, wherein the *P. fluorescens* type II asparaginase is expressed from a second expression construct

comprising a nucleic acid encoding the recombinant *P. fluorescens* type II asparaginase operably linked to the *P. fluorescens* AnsB secretion leader. In some examples, the second expression construct comprises a nucleic acid encoding the amino acid sequence set forth as SEQ ID NO: 55.

INCORPORATION BY REFERENCE

[0006] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The novel features of the disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0008] FIG. 1. SDS-CGE Gel-like Images - Tier 1 Expression Plasmid Screen. Asparaginase small scale (0.5 ml) growth whole broth sonicate soluble (upper panel) and insoluble (lower panel) were analyzed by reduced SDS-CGE. The lane at the far left shows molecular weight marker ladder (upper panel MW ladder 119 kDa, 68 KDa, 48 kDa, 29 kDa, 21 kDa, 16 kDa; lower panel MW ladder 119 kDa, 68 KDa, 48 kDa, 29 kDa, 21 kDa, 16 kDa) and the lane at the far right shows the same ladders. From left to right beginning immediately to the right of ladder 1 are lanes showing the expression patterns observed

CLAIMS

WHAT IS CLAIMED IS:

1. A method for producing a recombinant type II asparaginase, the method comprising: culturing a *Pseudomonadales* host cell deficient in the expression of at least one native asparaginase in a culture medium and expressing the recombinant type II asparaginase in the periplasm of the *Pseudomonadales* host cell from an expression construct comprising a nucleic acid encoding the recombinant type II asparaginase; wherein the recombinant type II asparaginase encoded by the nucleic acid comprises an amino acid sequence at least 85% identical to SEQ ID NO: 1, wherein the recombinant type II asparaginase is produced in the periplasm at a yield of about 33% to about 65% total cell protein (TCP) in soluble form.

2. The method of claim 1, wherein the soluble recombinant type II asparaginase is produced in the periplasm at a yield of about 10 g/L to about 38 g/L.

3. The method of claim 1, further comprising measuring the activity of an amount of the recombinant type II asparaginase produced, using an activity assay.

4. The method of claim 1, wherein the nucleic acid encoding the recombinant type II asparaginase is optimized for expression in the host cell.

5. The method of claim 1, wherein the recombinant type II asparaginase is an Escherichia coli L-asparaginase type II.

6. The method of claim 1, wherein the nucleic acid encoding the recombinant type II asparaginase comprises a sequence at least 85% identical to SEQ ID NO: 2 or 4.

7. The method of claim 1, wherein the recombinant type II asparaginase encoded by the nucleic acid comprises an amino acid sequence as set forth in SEQ ID NO: 1.

8. The method of claim 1, wherein the *Pseudomonadales* host cell is a *Pseudomonas fluorescens* cell.

9. The method of claim 8, wherein the one or more deficiently expressed native asparaginase is selected from: a type I asparaginase; a type II asparaginase; and
a combination thereof.

10. The method of claim 1, wherein the host cell is deficient in the expression of one or more proteases.

11. The method of claim 1, wherein the host cell overexpresses one or more folding modulators.

12. The method of claim 1, wherein the host cell is selected from:

a) a host cell that overexpresses LepB;

b) a host cell that overexpresses Tig;

c) a host cell that overexpresses DsbAC-Skp;

d) a host cell that is deficient in Lon, HslUV, DegP1, DegP2, AprA, DegP2 S219A, Prc1, or AprA;

e) a host cell that is deficient in AspG1;

f) a host cell that is deficient in AspG2;

g) a host cell that does not overexpress a folding modulator, and is not deficient in a protease;

h) a host cell that does not overexpress a folding modulator, is not deficient in a protease; and is not deficient in AspG1; and

i) a host cell that does not overexpress a folding modulator, is not deficient in a protease; and is not deficient in AspG2.

13. The method of claim 1, wherein the host cell is selected from:

a) a host cell that is deficient in Lon and HslUV;

b) a host cell that is deficient in Lon, DegP1, DegP2, Prc1, and AprA;

c) a host cell that is deficient in Lon, DegP1, DegP2 S219A, Prc1, and AprA, and overexpresses DsbAC-Skp;

d) a host cell that is deficient in AspG1 and/or AspG2;

e) a host cell that is deficient in AspG1 and/or AspG2, and overexpresses Tig;

f) a host cell that is deficient in AspG1 and/or AspG2, and overexpresses LepB;

g) a host cell that is deficient in AspG1 and/or AspG2, and deficient in Lon and HslUV; h) a host cell that is deficient in AspG1 and/or AspG2, and deficient in Lon, DegP1, DegP2, Prc1, and AprA; and

i) a host cell that is deficient in AspG1 and/or AspG2, Lon, DegP1, DegP2, Prc1, and AprA, and overexpresses DsbAC-Skp.

14. The method of claim 1, wherein the expression construct comprises a secretion leader that directs transfer of the recombinant type II asparaginase produced to the periplasm of the host cell.

15. The method of claim 14, wherein the secretion leader is selected from the group consisting of: *P. fluorescens* AnsB secretion leader, *P. fluorescens* 8484 secretion leader, *P. fluorescens* IBP-S**31**A secretion leader, *P. fluorescens* pbp secretion leader, *P. fluorescens* 8584 secretion leader, *P. fluorescens* LAO secretion leader, *P. fluorescens* Azu secretion leader, *P. fluorescens* PbpA20V secretion leader, *P. fluorescens* CupC2 secretion leader, and the *Escherichia coli* K-12 AnsB secretion leader.

16. The method of claim 3, further comprising comparing the measured activity of the recombinant type II asparaginase produced with an activity measured in the same amount of a control type II asparaginase using the same activity assay, wherein the measured activity of the recombinant type II asparaginase produced is comparable to the activity of the control type II asparaginase.

17. The method of claim 1, wherein the recombinant type II asparaginase produced is modified to increase half-life in patients.

18. The method of claim 15, wherein the recombinant type II asparaginase expressed from the expression construct is a recombinant *E. coli* type II asparaginase, wherein the nucleic acid encodes the recombinant E. coli type II asparaginase operably linked to the *P. fluorescens* AnsB secretion leader, wherein the recombinant *E. coli* type II asparaginase is produced in the periplasm at a yield that is about 20% to about 100% greater than that of a recombinant *P. fluorescens* type II asparaginase produced in the periplasm by the same method, and wherein the *P. fluorescens* type II asparaginase is expressed from a second expression construct comprising a nucleic acid encoding the recombinant *P. fluorescens* type II asparaginase operably linked to the *P. fluorescens* AnsB secretion leader.

19. The method of claim 18, wherein the second expression construct comprises a nucleic acid encoding the amino acid sequence set forth as SEQ ID NO: 55.

20. Use of the recombinant type II asparaginase produced by the method of claim 1 in the manufacture of a medicament for the treatment of patients with neoplastic conditions.

21. The use of claim 20, wherein the neoplastic condition is selected from: acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma.

22. The method of claim 17, wherein the recombinant type II asparaginase is modified to increase half-life in patients by pegylation to add at least one polyethylene glycol (PEG).

23. The method of claim 22, wherein the PEG is monomethoxy polyethylene glycol (mPEG).

24. The method of claim 16, wherein the control type II asparaginase is an *E. coli* type II asparaginase.

25. The recombinant type II asparaginase produced by the method of claim 1 for use in therapy.