

Description

BACKGROUND OF THE INVENTION

The present invention relates to extraction of granulocyte/macrophage colony stimulating factor (GM-CSF) from GM-CSF-expressing bacteria.

Granulocyte/macrophage colony stimulating factor is believed to be a potential therapeutic agent against infection and cancer. Clinical testing and widespread use of GM-CSF have been delayed owing to the unavailability of sufficient quantities of the material and the great expense of obtaining GM-CSF from natural sources. Recombinant DNA techniques have been used to create bacteria capable of expressing GM-CSF; see, for example, DeLamar et al., EMBO J., Vol. 4, 2575-2581 (1985). Fermentation of such bacteria is expected to yield sufficient quantities of GM-CSF at substantially lower cost than would be possible utilizing natural sources of GM-CSF. However, clinical use of GM-CSF also requires high purity material that is not contaminated by cell constituents or cell debris of the GM-CSF-expressing bacteria. Contamination by such impurities could result in adverse reactions or in test results that are not reproducible. Accordingly, extraction of GM-CSF from the cells of GM-CSF-expressing bacteria in sufficiently high purity and yield for clinical use has been a major problem.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that GM-CSF can be extracted from GM-CSF-expressing bacteria in high yield and purity by treating a suspension of GM-CSF-containing bacterial cells with an acid and an enhancing agent, or with an acid that is itself an enhancing agent, removing and discarding substantially all of the suspension liquid from the cells, preparing a second suspension of the treated cells, neutralizing said second suspension and separating the GM-CSF-containing liquid from the suspended cells. In accordance with the method of the present invention, GM-CSF is obtained from the cells without the need for mechanical or enzymatic disruption of the cell surface. The method of this invention allows recovery of GM-CSF in a manner which significantly reduces contamination by cell constituents, and subsequent purification is easier and less expensive.

The acid in the killing step is supplemented with an "enhancing agent" that increases the kill at a given pH and that also preferably helps the escape of GM-CSF from the cells.

The word "neutralizing" in the foregoing paragraph means that the second suspension is rendered approximately neutral (e.g. pH 6.0 to 8.0) or weakly alkaline (e.g. up to about pH 9.0).

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a construction map of plasmid pAKG-151.

DETAILED DESCRIPTION

The present invention provides a method for extracting GM-CSF from GM-CSF-expressing bacterial cells comprising:

- (a) treating a suspension of GM-CSF-containing bacterial cells with (i) an acid and an enhancing agent or (ii) an acid that is itself an enhancing agent so as to kill said cells, said enhancing agent causing killing of the cells at a higher pH than in the absence of said enhancing agent;
- (b) removing substantially all of the suspension liquid from the treated cells;
- (c) preparing a second suspension of the treated cells;
- (d) neutralizing said second suspension; and
- (e) separating the GM-CSF-containing liquid from the suspended treated cells.

In carrying out the method of the present invention, acid is added to a suspension of GM-CSF-expressing cells to adjust the pH to a lethal value for the cells, i.e. to about 1.5 to 3.0, preferably to about 2.0 to 2.2. Examples of suitable acids that can be utilized in this invention are hydrochloric acid, nitric acid, phosphoric acid and sulfuric acid. Phosphoric acid is the preferred acid.

It should be noted that acid alone at pH 3.0 will kill the bacteria, but the low pH required for complete kill (down to pH 1.5) can cause damage to the GM-CSF by denaturation or decomposition (e.g. deamination). However, the use of an "enhancing agent" provides complete kill of the bacteria at a higher pH, e.g. in particular 2.0 to 2.2, where the likelihood of damage to the GM-CSF is much lower. Killing all the bacteria at this stage is highly desirable to ensure containment of a genetically-engineered microorganism.

The enhancing agent can itself be an acid, e.g. trichloroacetic acid, when it may be the sole acid used in the killing step.

The use of an enhancing agent not only aids in killing the bacterial cells but also often serves to improve the yield of extracted GM-CSF. Examples of suitable enhancing agents include chaotropic ions (or compounds providing them), such as trichloroacetate, perchlorate, thiocyanate and guanidinium; non-chaotropic salts, such as sodium chloride, sodium phosphate; and non-ionic chaotropes such as urea. Chaotropic ions are the preferred enhancing agents. Trichloroacetate is the most preferred enhancing agent (about 0.1M to 2.0M depending on such variables as cell density, pH and salt composition).

When the enhancing agent is a chaotropic ion it may be added either as a chaotropic salt, such as

sodium thiocyanate or guanidinium chloride, or as a chaotropic acid, such as trichloroacetic acid or perchloric acid. If a chaotropic acid is used, then less or none of the other acid may be necessary.

In one embodiment of the acidification step of the method of this invention, phosphoric acid is added to the suspension to lower the pH to about 4 to 5, preferably 4.5, and then trichloroacetic acid is added to lower the pH to about 2.0.

If the temperature is too low then the bacteria will not be killed fast enough during the acid treatment. If the temperature is too high then GM-CSF may be altered. The temperature range for the acid treatment should be from about 10°C to about 40°C, and preferably about 25°C.

After treating the cell suspension with acid and also with an enhancing agent, all subsequent steps of the method of this invention are carried out at a temperature of from about 0°C to about 40°C, preferably 0° to 4°C.

After the cell suspension is treated with an acid (and an enhancing agent), to kill the bacterial cells, the cells are separated from the treatment liquid by microfiltration, centrifugation or the like, preferably by centrifugation, and resuspended in an aqueous buffer solution or in water. Examples of buffers that may be used in resuspending the pellet are sodium phosphate, potassium phosphate and tris (hydroxymethyl) aminomethane hydrochloride. Preferred buffers are sodium phosphate and especially tris (hydroxymethyl) aminomethane hydrochloride.

The cell suspension is neutralized to a pH of about 6.0 to 9.0, preferably 7.2 to 7.6. Examples of suitable bases that may be used in the neutralization step are sodium hydroxide, potassium hydroxide and the like.

Examples of bacteria that can be altered by recombinant DNA techniques to produce GM-CSF and from which GM-CSF may then be extracted using the method of the present invention are *E. coli*, *Bacillus subtilis*, *Streptomyces coelicolor*, and the like. The preferred bacterium is *E. coli*.

The method of the present invention may be used with bacteria that express different forms of GM-CSF, for example human GM-CSF [Lee et al., Proc. Natl. Acad. Sci. USA, Vol. 82, 4060-4064 (1985)], or murine GM-CSF [Burgess et al., J. Biol. Chem., Vol. 252, 1998-2033 (1977)].

The following Example describes the invention in detail. It will be apparent to those skilled in the art that modification of materials and methods may be practised without departing from the purpose and intent of this disclosure.

EXAMPLE

The human GM-CSF expression plasmid, pAKG-151 used in this example consists of about 3800 base

pairs and includes the following sequences (see Figure 1):

(a) The double tandem promoter lpp/lac linked to the ompA signal sequence; Ghayeb, et al., EMBO J., Vol. 3 (10), 2437-2442 (1984).

(b) The coding sequence for mature Hu-GM-CSF; See Lee et al., Proc. Natl. Acad. Sci. USA, Vol. 82, 4360-4364 (July 1985). The 5'-end of this coding sequence is fused with the 3'-end of the ompA signal coding sequence.

(c) The lac I gene for the expression of the lac repressor; Farabaugh, Nature, Vol. 274, 765-769 (Aug. 24, 1978).

(d) The temperature-sensitive replicon, rep cop Ts, derived from the plasmid pVU 208; Hakkart et al., Mol. Gen. Genet., Vol. 183, 326-332 (1981).

(e) The kan^r gene for the expression of aminoglycoside 3'-phosphotransferase II; Beck, et al., Gene 19, 327-336 (1982).

Cultivate a culture of *E. coli* strain 294 harboring the plasmid pAKG-151 in 200 ml of broth contained in a 2 liter baffled shake-flask at 30°C. The broth consists of 30 g/l of casein hydrolysate, 20 g/l of yeast extract, 20 g/l of glycerol, 10 mg/l of kanamycin, 5 g/l KH₂PO₄, 1 g/l MgSO₄·7H₂O, 0.1 ml/l of an antifoam agent, and water. The initial pH is adjusted to 7.0 with sodium hydroxide. Agitate until the cellular density of the culture reaches about 4 optical density units (light-path 1 cm, 660 nm). Add 0.4 mM of isopropyl-β-D-thiogalactoside and continue the fermentation for about 3 hours attaining cellular density of about 9 optical density units. Then add 85% phosphoric acid to pH 4.0 followed by 50% trichloroacetic acid to pH 2.0. Agitate the acidified suspension for 1 hour at 30°C, centrifuge the suspension, discard the supernatant and resuspend the bacterial pellet in 0.1M sodium phosphate buffer pH 8.5 or in Tris.HCl buffer pH 8.5. The pH of the resulting suspension is adjusted to pH 7.0 to 7.5 with 1M sodium hydroxide. Adjust the final biomass concentration of the neutral suspension to correspond to about 30 optical density units of untreated culture. Agitate the neutral suspension for 30 minutes at 4°C, centrifuge, and discard the pellet. The supernatant contains extracted recombinant human granulocyte macrophage colony stimulating factor (GM-CSF).

Claims

1. A method of extracting granulocyte macrophage colony stimulating factor (GM-CSF) from GM-CSF-containing bacterial cells comprising:

(a) treating a suspension of GM-CSF-containing bacterial cells with (i) an acid and an enhancing agent or (ii) an acid that is itself an enhancing agent, so as to kill said cells, said enhancing agent causing killing of the cells at a higher pH



sodium thiocyanate or guanidinium chloride, or as a chaotropic acid, such as trichloroacetic acid or perchloric acid. If a chaotropic acid is used, then less or none of the other acid may be necessary.

In one embodiment of the acidification step of the method of this invention, phosphoric acid is added to the suspension to lower the pH to about 4 to 5, preferably 4.5, and then trichloroacetic acid is added to lower the pH to about 2.0.

If the temperature is too low then the bacteria will not be killed fast enough during the acid treatment. If the temperature is too high then GM-CSF may be altered. The temperature range for the acid treatment should be from about 10°C to about 40°C, and preferably about 25°C.

After treating the cell suspension with acid and also with an enhancing agent, all subsequent steps of the method of this invention are carried out at a temperature of from about 0°C to about 40°C, preferably 0° to 4°C.

After the cell suspension is treated with an acid (and an enhancing agent), to kill the bacterial cells, the cells are separated from the treatment liquid by microfiltration, centrifugation or the like, preferably by centrifugation, and resuspended in an aqueous buffer solution or in water. Examples of buffers that may be used in resuspending the pellet are sodium phosphate, potassium phosphate and tris (hydroxymethyl) aminomethane hydrochloride. Preferred buffers are sodium phosphate and especially tris (hydroxymethyl) aminomethane hydrochloride.

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- than in the absence of said enhancing agent;
- (b) removing substantially all of the suspension liquid from the cells;
- (c) preparing a second suspension of the treated cells;
- (d) neutralizing said second suspension; and
- (e) separating the GM-CSF-containing liquid from the suspended cells.

2. The method according to claim 1, wherein said suspension in step (a) is acidified to a pH of about 1.5 to 3.0, preferably about 2.0 to 2.2.

3. The method according to claim 1, wherein said suspension in step (a) is treated with hydrochloric acid, nitric acid, sulfuric acid, or preferably phosphoric acid.

4. The method according to claim 1, wherein the enhancing agent is a chaotropic ion selected from perchlorate, thiocyanate, guanidinium, and especially trichloroacetate.

5. The method according to claim 1 wherein the enhancing agent is a non-chaotropic salt selected from sodium chloride and sodium phosphate, or urea.

6. The method according to claim 1, wherein step (a) is carried out at a temperature from about 10°C to 40°C, preferably about 25°C.

7. The method according to claim 1, wherein said second suspension is neutralized with sodium hydroxide.

8. The method according to claim 1, wherein the second suspension is neutralized to a pH of about 6 to 9, preferably about 7.2 to 7.6.

9. The method according to claim 1, wherein steps (b) through (e) are carried out at a temperature of from about 0° to 40°C, preferably about 0° to 4°C.

10. The method according to claim 1, wherein the bacterium is selected from Bacillus subtilis, Streptomyces coelicolor, and especially Escherichia coli.

Patentansprüche

1. Verfahren zum Extrahieren eines Granulocyt-Makrophagen-Koloniestimulierungsfaktors (GM-CSF) aus GM-CSF enthaltenden Bakterien-Zellen, umfassend

- (a) das Behandeln einer Suspension von GM-CSF enthaltenden Bakterienzellen mit (i) einer Säure und einem Verstärkungsmittel oder (ii) einer Säure, die selbst ein Verstärkungsmittel ist, um die Zellen abzutöten, wobei das Verstärkungsmittel so wirkt, daß es der Säure die Abtötung der Zellen bei einem höheren pH-Wert als in Abwesenheit des Verstärkungsmittels ermöglicht;
- (b) das Entfernen der im wesentlichen vollständigen Menge der Suspensionsflüssigkeit von den Zellen;
- (c) das Herstellen einer zweiten Suspension der

behandelten Zellen;

(d) das Neutralisieren der zweiten Suspension; und

(e) das Abtrennen der GM-CSF enthaltenden Flüssigkeit von den suspendierten Zellen.

2. Verfahren nach Anspruch 1, worin die Suspension in Schritt (a) auf einen pH-Wert von etwa 1,5 bis 3,0, vorzugsweise von etwa 2,0 bis 2,2, angesäuert wird.

3. Verfahren nach Anspruch 1, worin die Suspension in Schritt (a) mit Salzsäure, Salpetersäure, Schwefelsäure oder vorzugsweise Phosphorsäure behandelt wird.

4. Verfahren nach Anspruch 1, worin das Verstärkungsmittel ein chaotropes Ion, ausgewählt aus Perchlorat, Thiocyanat, Guanidinium und insbesondere Trichloroacetat, ist.

5. Verfahren nach Anspruch 1, worin das Verstärkungsmittel ein nicht-chaotropes Salz, ausgewählt aus Natriumchlorid und Natriumphosphat, oder Harnstoff ist.

6. Verfahren nach Anspruch 1, worin der Schritt (a) bei einer Temperatur von etwa 10 °C bis 40 °C, vorzugsweise bei etwa 25 °C, durchgeführt wird.

7. Verfahren nach Anspruch 1, worin die zweite Suspension mit Natriumhydroxid neutralisiert wird.

8. Verfahren nach Anspruch 1, worin die zweite Suspension auf einen pH-Wert von etwa 6 bis 9, vorzugsweise von etwa 7,2 bis 7,6, neutralisiert wird.

9. Verfahren nach Anspruch 1, worin die Schritte (b) bis (e) bei einer Temperatur von etwa 0 °C bis 40 °C, vorzugsweise bei etwa 0 °C bis 4 °C, durchgeführt werden.

10. Verfahren nach Anspruch 1, worin das Bakterium aus Bacillus subtilis, Streptomyces coelicolor und insbesondere Escherichia coli, ausgewählt wird.

Revendications

1. Procédé d'extraction du facteur (GM-CSF) de stimulation de colonies de granulocytes macrophages, de cellules bactériennes contenant GM-CSF, consistant à :

- (a) traiter une suspension de cellules bactériennes contenant GM-CSF avec (i) un acide et un agent activant ou (ii) un acide qui est lui-même un agent activant, de façon à tuer lesdites cellules, ledit agent activant étant efficace pour permettre à l'acide de tuer des cellules à un pH plus élevé qu'en l'absence dudit agent activant ;
- (b) éliminer sensiblement tout le liquide de suspension des cellules ;
- (c) préparer une deuxième suspension des cellules traitées ;
- (d) neutraliser ladite deuxième suspension ; et
- (e) séparer le liquide contenant GM-CSF des cellules en suspension.



2. Procédé suivant la revendication 1, caractérisé en ce que ladite suspension dans l'étape (a) est acidifiée à un pH d'environ 1,5 à 3, de préférence environ 2 à 2,2.

3. Procédé suivant la revendication 1, caractérisé en ce que ladite suspension dans l'étape (a) est traitée avec de l'acide chlorhydrique, de l'acide nitrique, de l'acide sulfurique, ou préférentiellement de l'acide phosphorique.

4. Procédé suivant la revendication 1, caractérisé en ce que l'agent activant est un ion chaotropique choisi parmi perchlorate, thiocyanate, guanidinium, et en particulier trichloroacétate.

5. Procédé suivant la revendication 1, caractérisé en ce que l'agent activant est un sel non-chaotropique choisi parmi le chlorure de sodium et le phosphate de sodium, ou l'urée.

6. Procédé suivant la revendication 1, caractérisé en ce que l'étape (a) est réalisée à une température d'environ 10°C à 40°C, de préférence environ 25°C.

7. Procédé suivant la revendication 1, caractérisé en ce que ladite deuxième suspension est neutralisée avec de l'hydroxyde de sodium.

8. Procédé suivant la revendication 1, caractérisé en ce que la deuxième suspension est neutralisée à un pH d'environ 7 à 9, de préférence d'environ 7,2 à 7,6.

9. Procédé suivant la revendication 1, caractérisé en ce que les étapes (b) à (e) sont réalisées à une température d'environ 0° à 40°C, de préférence d'environ 0° à 4°C.

10. Procédé suivant la revendication 1, caractérisé en ce que la bactérie est choisie parmi Bacillus subtilis, Streptomyces coelicolor, et en particulier Escherichia coli.

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