

DESCRIPTION

TITLE

CELL-GROWING CULTURE MEDIUM FOR PRODUCING CULTURED MEAT

5

FIELD

[0001]

The present invention relates to the technical field of producing cultured meat. More specifically, the invention relates to a cell proliferation medium to be used for producing
10 cultured meat, a method for producing the medium, a method for preparing cells for producing cultured meat, and a cell growth promoting agent for producing cultured meat.

BACKGROUND

[0002]

15 Meat has traditionally been produced by raising livestock. Raising of livestock, however, requires large amounts of cereal feed and water, as well as large breeding farms. In recent years, awareness of problems such as climate change and food shortages has led to demand for more sustainable meat production with a goal toward reduced environmental impact and higher productivity. In light of this situation, increasing attention is being focused on production of
20 cultured meat from cells, as a novel method of meat production.

[0003]

Plant-derived meat substitutes are known, but they still fail to provide the mouthfeel and flavor of meat. Cultured meat obtained by culturing of animal cells, however, can provide a mouthfeel and flavor similar to that of conventional meat, with the added advantage of lower risk
25 of contamination by bacteria and viruses than conventional meat. Production of cultured meat has recently become technically feasible. However, since the cell culture media currently used for large-scale culture techniques employed for basic research and drug application not for producing cultured meat, they are difficult to use for food production due to issues of cost and safety. For cell culture media used for basic research and drug applications it is common to add
30 fetal bovine serum (FBS) as an added component to basal medium, containing carbon sources such as amino acids, vitamins, inorganic salts and glucose. Because FBS is fetus-harvested serum, it is difficult to obtain in mass quantities and it is also associated with issues of basic cost, transport cost, infectious disease risk and animal welfare. (NPL 1: ALTEX. 2018; 35(1):99-118). To overcome these issues, completely synthetic media have been developed as reagents that
35 provide the essential components of FBS (NPL 2: The Canadian Journal of Chem Engineering 2016, Vol. 94, (10) 1855-1862). Nevertheless, such completely synthetic media use recombinant

proteins, steroid hormones and serum-derived components, which are problematic for use in foods.

[0004]

The use of cell culture media for cultured meat production has been attempted using various approaches. These include media utilizing organ cell products (PTL 1: Japanese Patent Publication No. 6111510), media utilizing algae products (NPL 3: Scientific Reports. 2017; 7:41594), media utilizing food residue hydrolysates (NPL 4: Food Funct., 2020, 11, 2477-2488), and media utilizing other food material components (PTL 2: International Patent Publication No. WO2021/148955, NPL 5: Jpn J Exp Med. 1985 Apr; 55(2):45-51, NPL 6: Prog Immunobiol Stand. 1971; 5:202-8, NPL 7: BMC Biotechnology. 2023 Feb 8; 23(1):4).

[CITATION LIST]

[PATENT LITERATURE]

[0005]

[PTL 1] Japanese Patent Publication No. 6111510

[PTL 2] International Patent Publication No. WO2021/148955

[NON PATENT LITERATURE]

[0006]

[NPL 1] ALTEX. 2018; 35(1):99-118.

[NPL 2] The Canadian Journal of Chem Engineering 2016, Vol. 94, (10) 1855-1862

[NPL 3] Scientific Reports. 2017; 7:41594

[NPL 4] Food Funct. 2020, 11, 2477-2488

[NPL 5] Jpn J Exp Med. 1985 Apr; 55(2):45-51

[NPL 6] Prog Immunobiol Stand. 1971; 5:202-8

[NPL 7] BMC Biotechnology . 2023 Feb 8; 23(1):4.

SUMMARY

[TECHNICAL PROBLEM]

[0007]

The object of the present invention is to provide a culture medium which allows large-scale culturing of cells to be used for production of cultured meat, by addition of food material components as cell growth promoting agents.

[SOLUTION TO PROBLEM]

[0008]

The present inventors have ardently studied culture media usable for production of cultured

meat, and have completed this invention upon finding that adding egg white or dried egg white as a cell growth promoting agent to a medium allows high proliferative activity for cells used for cultured meat. The present invention relates to the following:

[1] A cell proliferation medium containing basal medium and egg white or dried egg white as a cell growth promoting agent.

[1-2] The use of egg white or dried egg white for production of a cell proliferation medium.

[1-3] The use of egg white or dried egg white for production of a cell growth promoting agent.

[2] The medium according to [1], or the use according to [1-2], wherein the proliferated cells are cells to be used for producing cultured meat.

[3] The medium according to [1] or [2], or the use according to [1-2], [1-3] or [2], wherein the medium does not contain animal-derived serum.

[4] The medium according to [3] or its use, wherein the animal-derived serum is fetal bovine serum (FBS).

[5] The medium according to any one of [1] to [4], or the use according to [1-2], [1-3] or [2-4], wherein the cells include fibroblasts or muscle tissue cells.

[6] The medium according to any one of [1] to [5], or the use according to [1-2], [1-3] or [2-5], wherein the cells are cells derived from a cow, pig or chicken.

[7] A method for preparing cells for producing cultured meat, the method comprising a step of culturing cells in medium containing basal medium and egg white or dried egg white as a cell growth promoting agent.

[8] The method according to [7], wherein the medium does not contain animal-derived serum.

[9] The method according to [8], wherein the animal-derived serum is fetal bovine serum (FBS).

[10] The method according to any one of [7] to [9], wherein the cells are cells derived from a cow, pig or chicken.

[11] The method according to [10], wherein the cells include at least one type of cell selected from the group consisting of fibroblasts and muscle tissue cells.

[12] A method for producing cultured meat, the method comprising a step of accumulating cells prepared by the method according to any one of [7] to [11].

[13] The method according to [12], wherein the prepared cells are accumulated together with one or more substances selected from the group consisting of other cells, blood, tissue and extracellular matrix.

[14] The method according to [13], wherein the other cells are cultured cells or cells acquired from an animal.

[15] The method according to any one of [12] to [14], which includes further culturing after accumulation.

[16] A cell growth promoting agent for producing cultured meat, which comprises egg white or dried egg white.

[17] The cell growth promoting agent according to [16], which is added to animal-derived serum-free medium.

[18] The cell growth promoting agent according to [16] or [17], which promotes growth of at least one type of cell selected from the group consisting of fibroblasts and muscle tissue cells.

[19] The cell growth promoting agent according to [18], wherein the cells are cells from a cow, pig or chicken.

[ADVANTAGEOUS EFFECTS OF INVENTION]

[0009]

By adding egg white or dried egg white to basal medium it is possible to formulate a cell culture medium that promote enhanced cell growth.

BRIEF DESCRIPTION OF DRAWINGS

[0010]

Fig. 1 shows the results of screening for food material components that enhance cell proliferation, when culturing bovine fibroblasts (A) and bovine myoblasts (B) in serum-free medium.

Fig. 2 shows the results of screening for food material components that enhance cell proliferation, when culturing chicken fibroblasts (A) and chicken myoblasts (B) in serum-free medium.

Fig. 3 shows cell proliferation rates with respect to cell counts for culturing pig fibroblasts in serum-free medium, with culturing in dried egg white-added medium and FBS-added medium.

Fig. 4 is a graph showing the concentration-dependent growth promoting effect when using egg white as a cell growth promoting agent when culturing chicken fibroblasts (A) and pig fibroblasts (B) in serum-free medium.

Fig. 5 shows the results of molecular weight analysis of an egg white sample after freeze-drying.

DESCRIPTION OF EMBODIMENTS

[0011]

The present invention relates to a cell proliferation medium containing basal medium and

egg white or dried egg white as a cell growth promoting agent. According to a different aspect, the invention relates to a method of preparing cells for producing cultured meat, the method including a step of culturing cells in cell proliferation medium containing basal medium and egg white or dried egg white as a cell growth promoting agent, and to a method for producing cultured meat from the prepared cells. According to yet another aspect, the invention relates to a cell growth promoting agent for producing cultured meat which contains egg white or dried egg white.

[0012]

[Cell proliferation medium]

The cell proliferation medium of the invention contains basal medium and egg white or dried egg white as a cell growth promoting agent. Because egg white or dried egg white is a food material, cells cultured in the medium of the invention are highly safe for foods. Moreover the low cost of egg white as a raw material provides the advantage of lower cost for preparation of the medium of the invention. Moreover, egg white or dried egg white can enhance cell growth activity. The egg white or dried egg white may be added at a relatively low concentration, such as 0.001 to 0.5 mass% and preferably 0.003 to 0.2 mass%, to enhance the cell growth activity. Addition at low concentration will inhibit aggregation and facilitate preparation of the medium. Cells cultured in such medium are highly safe for foods and can therefore be used for producing cultured meat. The medium of the invention is serum-free medium that, while including egg white or dried egg white, is also free of animal-derived serum.

[0013]

Animal-derived serum is serum produced from animal blood. The supernatant liquid obtained by coagulating harvested blood is referred to as “serum”. Animal-derived serum may be serum derived from any animal such as a cow, horse, goat, donkey, rabbit or bird, but it may particularly refer to bovine serum (BCS) or fetal bovine serum (FBS). Serum includes proteins such as albumin and globulin, and serum lipids such as triglycerides, cholesterol, phospholipids and free fatty acids, as well as hormones, cytokines and growth factors. Fetal serum contains abundant amounts of components necessary for cell proliferation and is commonly added to medium in the fields of research and medicine. Medium lacking animal-derived serum is referred to as “serum-free medium”. Serum-free medium lacks animal-derived serum but may contain purified components derived from serum, or recombinant proteins from serum-derived components.

[0014]

According to the invention it is possible to obtain high cell growth activity even with serum-free medium, by addition of egg white or dried egg white to basal medium. The egg white of the invention may therefore be considered to be a cell growth promoting agent (also referred

to as “cell culture supplement”). The cell growth promoting agent of the invention can be used in cell culturing for production of cultured meat, and may be added to animal-derived serum-free medium.

[0015]

5 Egg white is a substance obtained by separating out the shells and the yolk in bird's eggs. Any type of bird may be used, but from the viewpoint of availability for large-scale use, it may be poultry, such as chicken, quail, turkey, domesticated duck, goose or ostrich. The egg white may be either an isolated product from the egg or a dried isolated product. A dried product is usually provided as a dry powder. A dried egg white product is preferred to reduce transport
10 costs. The dried product may be prepared by any desired drying method, but it is more preferably prepared by freeze-drying. The dried egg white used may be a commercially available product, or it may be prepared by drying egg white. After the egg white or dried egg white has dissolved in the medium, it may be filtered using a filter with a suitable pore size to remove the insoluble matter. For example, filtration may be carried out using a 0.3 to 0.5 μm and especially a 0.45 μm
15 filter. When dried egg white is to be used as a growth promoting agent, it is added at a concentration appropriately selected to increase the proliferation rate, depending on the type of cells used. For example, it may be added to the basal medium at 0.001 mass% to 0.5 mass%. The concentration of the egg white is preferably 0.002 mass% or greater and more preferably 0.003 mass% or greater from the viewpoint of exhibiting a proliferation effect. It is also preferably 0.2
20 mass% or lower and more preferably 0.1 mass% or lower from the viewpoint of achieving plateau of the growth promoting effect, or preventing its reduction. When an isolated product is used, the amount of addition may be determined in terms of the dried product.

[0016]

Egg white is composed mainly of various proteins, examples of which include ovalbumin,
25 ovomucoid, ovomucin, globulin, lysozyme and avidin. Trace components such as free amino acids, inorganic salts and vitamins may also be included. Egg white or dried egg whites can be characterized as having an average molecular weight of 5000 to 800,000 Da. The average molecular weight will vary depending on treatment of the egg white, but it most preferably has an average molecular weight of 10,000 Da or greater, 15,000 Da or greater or
30 18,000 Da or greater, from the viewpoint of ensuring that hydrolysis, and especially enzymatic hydrolysis and chemical hydrolysis, do not take place.

[0017]

Basal medium is medium for cell culturing, containing the minimum necessary components for maintenance and growth of the cells. By seeding the cells in basal medium it is possible to
35 sustain them without dying, allowing the cells to proliferate. A number of options are commercially available as basal media, but most generally contain amino acids, vitamins,

buffers, inorganic salts and carbon sources. Essential amino acids and non-essential amino acids are both included as amino acids. Vitamin B1, vitamin C, nicotinic acid and folic acid are included as vitamins. HEPES is included as a buffer. Carbon sources that may be added are monosaccharides such as glucose, disaccharides such as sucrose, oligosaccharides, and polysaccharides. Cell culture medium can generally be prepared by addition of additives such as serum to basal medium. The basal medium used may be any basal medium known in the technical field, examples of which include Dulbecco's Modified Eagle's Medium (DMEM), Basal Medium Eagle (BME), RPMI 1640 medium, DMEM/F12 medium, F10 medium, F12 Ham's Medium, MEM, M199 medium, Ames medium, Iscove's modified medium, Glasgow modified medium and Fischer's medium.

[0018]

A cell growth promoting agent is added to basal medium for cell culturing. For conventional cell culturing, serum such as fetal bovine serum (FBS) is added as a cell growth promoting agent. The cell proliferation medium of the invention, however, contains egg white or dried egg white as a cell growth promoting agent. The cell proliferation medium of the invention is therefore free of animal-derived serum, instead comprising egg white or dried egg white as a substitute, with the egg white or dried egg white functioning as a serum substitute. According to the invention, other additives different from the egg white or dried egg white may also be added to the medium. Such additives include components known to be added to serum-free medium in the technical field. Examples of additives added to serum-free medium in the technical field include lipids, hormone agents, growth factors, cytokines, serum-derived proteins and antibiotics. Hormone agents include dexamethasone. Growth factors include FGF, IGF and insulin, or any of their respective families. Cytokines include IL-1 α and IL-1 β , which may be added at a concentration of 0.1 to 1000 ng/mL, for example. Serum-derived proteins include fetuin, fibronectin, albumin and globulin, which may be added at a concentration of 0.0001 to 1%, for example. Antibiotics include penicillin and streptomycin which may be added at a concentration of 10 to 500 U/ml for penicillin or 10 to 500 μ g/mL for streptomycin, for example. ITS (insulin-transferrin-sodium selenite), an additive commonly used in serum-free media or low-serum media, may also be added to the egg white or dried egg white-containing serum-free medium of the invention. It may be added in an amount for a concentration of 0.1 to 5% as a 100-fold concentration premix solution, for example.

[0019]

When egg white or dried egg white is to be added to basal medium as a cell growth promoting agent, other food material components may be added as well. Optional components may also be added so long as effects suitable for cell culturing are exhibited. Effects appropriate for cell culturing include an effect of promoting or inhibiting differentiation, of a growth

promoting effect, for example. As an example, components with higher cell growth activity are preferred over addition of egg white or dried egg white alone, and soybean, wheat, fish powder or microorganisms, such as *Chlorella* components, may be added. Such food components may be added as extracts, or they may be added as dry powders with filtered removal of the insoluble components. These food materials are added to basal medium at 0.0025 mass% to 1.0 mass%, and from the viewpoint of exhibiting a growth effect, they are preferably added to the basal medium at 0.005 mass% or greater and more preferably 0.01 mass% or greater. From the viewpoint of avoiding aggregation of the components, the amount is also preferably 0.5 mass% or lower and more preferably 0.1 mass% or lower. The mass ratio of egg white or dried egg white to the other food material components may be appropriately selected in a range of 10:1 to 1:10. The range is preferably 5:1 to 1:5 and more preferably 3:1 to 1:3.

[0020]

[Cells]

The cell culture medium of the invention allows culturing of any type of animal cells. From the viewpoint of cultured meat production, cells derived from livestock such as cows, pigs, goats, sheep, rabbits, chickens, ostriches or wild ducks may be used. It is particularly preferred to use cells of cow, pig or chicken. The cell culture medium of the invention also allows culturing of tissues as aggregates of cells. The animal cells may be primary cells harvested from animals, or subcultured cells obtained by subculturing of primary cells, or an established cell line.

Primary cells can be harvested by chopping animal tissue in medium. The cells may also be cells that have been differentiated from stem cells such as somatic stem cells, embryonic stem cells or induced pluripotent stem cells. From the viewpoint of cultured meat production, the culturing is preferably of one or more types of cells selected from the group consisting of fibroblasts, adipocytes and muscle tissue cells.

[0021]

Fibroblasts compose connective tissue which produce components of the extracellular matrix such as collagen and elastin. Muscle fibroblasts are referred to as “myofibroblasts”. Myofibroblasts form connective tissue surrounding muscular fiber bundles in skeletal muscle. Myofibroblasts express α -SMA, and produce the extracellular matrix while also being able to accumulate fat, thus contributing to food chewiness and taste.

[0022]

Muscle tissue cells are cells composing muscular tissue, and they are cultured after isolation from muscular tissue. Muscle tissue cells include myoblasts, muscle satellite cells and myotube cells. Especially, myoblasts and/or muscle satellite cells being preferred from the viewpoint of proliferation since myotube cells do not have proliferative ability. Muscle satellite cells are intramuscular somatic stem cells which can proliferate and differentiate into myoblasts.

Myoblasts are muscular fiber-derived mononuclear cells, which have proliferative ability. Upon differentiation of myoblasts, the myoblasts fuse together forming multinucleated myotube cells which further mature into muscular fibers. The structural units of muscular fibers are myofibrils, composed of the muscular proteins of actin fibers and myosin fibers, and depending on the isoform of the myosin they are classified as either red muscle fibers (type I and type IIA) or white muscle fibers (IIB), which also contribute to the taste of meat.

[0023]

Adipocytes are cells having fat droplets in the cytoplasm, and they are largely classified as white adipocytes or brown adipocytes, also including adipose stem cells as their precursor cells.

Adipocytes carry out biosynthesis from glucose to fatty acids, while also incorporating fatty acids by the fatty acid transport pathway, resulting in maturation and enlargement. In production of cultured meat, taste can be improved by accumulating adipocytes together with fibroblasts and muscle tissue cells.

[0024]

When cow cells are used, egg white or dried egg white may be used particularly for culturing of fibroblasts and myoblasts. When fibroblasts were cultured in the medium developed in the present invention, the relative growth rate exceeded 50% compared to that in 10% FBS, which was higher compared to that in non-added negative control, and that in soybean, bonito flakes or wheat contained media (Fig. 1A). When myoblasts were cultured, the relative growth rate in the medium developed in the present invention was 34% compared to that in 10% FBS, which was higher compared to the that in non-added negative control, and was higher than that in using soybean, bonito flakes or wheat contained one (Fig. 1B). The use of an enzyme-treated egg white preparation instead of FBS for culturing of bovine myoblasts has been disclosed (NPL 4). This method is convenient for the purpose of the invention from the viewpoint of allowing growth of bovine fibroblasts and myoblasts whether using egg white directly, or after treatment by drying alone. For culturing of bovine fibroblasts and myoblasts, ITS may be further added in addition to the egg white or dried egg white.

[0025]

When chicken cells are used, egg white or dried egg white may be used particularly for culturing of fibroblasts and myoblasts. When fibroblasts were cultured, the relative growth rate is at least 80% compared to using 10% FBS, and depending on their concentration, the relative growth rate is equivalent to or greater than that obtained with 10% FBS addition. This was significantly increased when using white egg white or dried egg white compared to the unadded negative control, and the relative growth rate was also found to be higher than when using wheat, rice, corn or wheat protein (Fig. 2A). When chicken fibroblasts were cultured using soybean or *Chlorella*, they exhibited the same relative growth rate as with addition of 10% FBS, indicating

that these components can be used as cell growth promoting agents. When myoblasts were cultured using egg white or dried egg white, they exhibited a relative growth rate exceeding 70% compared to using 10% FBS. The relative growth rate was increased compared to the relative growth rate of the unadded negative control, and they were higher than the relative growth rates obtained using wheat, rice bran, chickpea, bonito, fish gelatin, shrimp, whey, pig gelatin and the unadded negative control (Fig. 2B). The use of egg white or egg yolk fraction instead of FBS for culturing of chicken skin cells has been disclosed (NPL 5: Jpn J Exp Med. (1985). The invention is characterized by growing fibroblasts and/or myoblasts for the purpose of producing cultured meat. FBS is usually not very substitutable when culturing chicken myoblasts, but egg white or dried egg white exhibits a sufficient growth promoting effect as a substitute for FBS.

[0026]

Egg white or dried egg white may be used for culturing pig cells, and especially for culturing of fibroblasts. When fibroblasts have been cultured, they have a relative growth rate of at least 75% compared to using 10% FBS. Using egg white or dried egg white causes a significant increase compared to the non-added negative control (Fig. 3).

[0027]

[Cultured meat]

Cultured meat is edible meat produced by cell culturing. The phrase “for producing cultured meat” as used herein means that the method is to be used for production of cultured meat and so must be acceptable in terms of food hygiene. In terms of food hygiene it is preferable to avoid the use of animal-derived serum, hormone agents and gene recombinant proteins. The term “edible meat” generally refers to muscular fibers, connective tissue and fat aggregates. Cultured meat, on the other hand, while preferably modeling the structure of meat, does not necessarily need to include the entire structure of the meat, and it may include cultured products of fibroblasts, muscle tissue cells or adipocytes. The cultured product more preferably includes multiple types of cells. The cultured meat may also include the extracellular matrix in addition to cultured fibroblasts or muscle tissue cells. The method for producing cultured meat includes, for example:

a step of culturing at least one type of cultured cells selected from the group consisting of fibroblasts and muscle tissue cells, and

a step of collecting and accumulating the cultured cells. The method for producing cultured meat may also further comprise a differentiation-inducing step and a post-accumulation culturing step. The present invention relates to cultured meat comprising cells cultured in the cell proliferation medium of the invention.

[0028]

The cell culturing is carried out by seeding cells in the cell proliferation medium of the

invention, i.e. medium containing basal medium and egg white or dried egg white as a cell growth promoting agent. The culturing is conducted under conditions known in the technical field, such as in a CO₂ incubator at 37°C. The culturing may be attachment culture or suspension culture. The cultured cells can be collected as the cultured product by trypsin treatment, and may be further subcultured after collection. Culturing of the cells may be seeding and culturing of the cells on a releasable structure. The structure on which the proliferated cells have attached may also be collected as the cultured product. Such a structure may be formed of extracellular matrix components such as collagen, elastin, fibronectin, laminin and entactin, accumulating a cell-adhering structure to form cultured meat.

[0029]

The accumulation step includes forming a cultured product of one or more types of collected cells. The cultured product formed in the accumulation step may be a single piece of meat such as steak, or whole meat cuts or minced meat. The accumulation step includes accumulation of the cultured cell product together with one or more substances selected from the group consisting of other cells, blood and tissue. Other cells may be cultured cells or cells harvested from an animal. More specifically, the meat may be formed with a combination of other cells cultured in cell proliferation medium according to the invention. As an example, muscle tissue cells cultured in cell proliferation medium of the invention may be accumulated with adipose tissue cells and/or fibroblasts cultured in cell proliferation medium of the invention. Co-culturing may also be carried out after accumulation. As an example, cultured products of one or more different collected cells may be mixed and then seeded and co-cultured on an extracellular matrix. The extracellular matrix used may be collagen, elastin, fibronectin, laminin or entactin. The medium used in this case may also be cell proliferation medium of the invention.

[0030]

The accumulation step may be accumulation of cultured products of one or more collected cells with blood and/or tissue. The tissue may be harvested from animals or cultured tissue. As one example, cultured meat may be produced by accumulating blood, adipose tissue or muscular tissue separated during the process of treating meat, together with a cultured product.

[0031]

The differentiation-inducing step may be carried out after cell culturing, or before the accumulation step, during the accumulation step or after the accumulation step. A differentiation-inducing step allows differentiation of mononuclear muscle satellite cells and myoblasts to multinucleated myotube cells, and further maturation to muscular fibers, or intracytoplasmic build-up of fat droplets. Differentiation may be induced by a method known in the technical field, one example being a method of culturing under a high carbon dioxide concentration, such as culturing in a 5 to 10% (v/v) CO₂ atmosphere, to promote differentiation

to myotube cells.

[0032]

[Method for producing medium]

The cell proliferation medium of the invention is prepared by a production method comprising the following steps:

a step of mixing basal medium with egg white or dried egg white as a cell growth promoting agent to obtain a cell proliferation medium; and
a step of sterilizing the medium.

The production method of the invention may also include a step of heat sterilization or filter sterilization of components that are susceptible to heat denaturation, and subsequent addition to the medium.

[0033]

All of the publications mentioned throughout the present specification are incorporated herein in their entirety by reference. The Examples of the invention described below are intended to serve merely as illustration and do not limit the technical scope of the invention. The technical scope of the invention is limited solely by the description in the Claims. Modifications of the invention, such as additions, deletions or substitutions to the constituent features of the invention, are possible so long as the gist of the invention is maintained.

EXAMPLES

[0034]

Example 1: Preparation of cells

(1) Harvesting of bovine myoblasts

Bovine myoblasts were harvested by the following procedure. Harvested muscular tissue was washed with ethanol and phosphate buffered saline (PBS), and then scissors were used on a clean bench for fine chopping. The muscular tissue was digested by shake culturing for 1.5 hours at 37°C in Dulbecco's Modified Eagle Medium (DMEM) with addition of 0.2% collagenase II (Worthington). The reaction was suspended by addition of 20% FBS to the digested reaction mixture. After centrifugal separation of the digested solution at 80 × g for 3 minutes, the floating tissue was removed with forceps, and the supernatant liquid was separated off. The supernatant liquid obtained by further centrifugal separation at 80 × g for 3 minutes was passed through a cell sorting nylon mesh (100 μm). The filtrate was centrifuged at 1500 g for 5 minutes to obtain a precipitate, which was suspended in Dulbecco's Modified Eagle Medium containing 20% FBS. The cell suspension was passed through a 100 μm nylon mesh and then again through a 40 μm nylon mesh, and the filtrate was centrifuged at 1500 × g for 5 minutes. The precipitate was allowed to stand for 5 minutes on ice in red blood cell lysate and the blood cells were removed.

After washing twice with phosphate buffer, it was pooled in DMEM medium containing 20% FBS and 4 ng/mL human basic fibroblast growth factor (bFGF), and seeded in a culture dish, and the proliferated cells were used for testing.

[0035]

5 (2) Harvesting of bovine and pig fibroblasts

Fibroblasts were harvested by the following steps from skin tissue of livestock cows and livestock pigs. The tissue was washed with ethanol and PBS, and then the dermis layer was peeled off and isolated on a clean bench. The isolated tissue was finely chopped with scissors and allowed to stand on a culture dish containing DMEM medium with addition of 10% FBS, and then cultured for several days in a CO₂ incubator at 37°C. The migrated cells were recovered and used for the test.

[0036]

(3) Harvesting of chicken myoblasts

Fibroblasts were harvested from 18th-day chicken fertilized egg embryos, by the following procedure. After mincing breast tissue with scissors, it was placed in DMEM medium containing 2 mg/mL collagenase II (Sigma) and subjected to enzyme reaction for 1 hour at 37°C. After then suspending the tissue with a 10 mL syringe equipped with an 18G needle, the suspension was passed through a 100 µm filter. The filtered suspension was centrifuged at 1500 rpm for 5 minutes, and the precipitate was suspended in DMEM medium containing 20% FBS and 4 ng/mL bFGF. The cell suspension was seeded in a tissue culture dish. After one night of culturing, the non-adherent cells were seeded in a collagen-coated culture dish and subcultured twice for use as myoblasts.

[0037]

Example 2: Search for food materials that promote cell growth in serum-free medium

25 (1) Bovine cells

Livestock bovine myoblasts and fibroblasts were used to search for components that promote growth in serum-free medium. The serum-free medium used was DMEM medium with addition of 1% penicillin-streptomycin (PS) solution, 1% ITS liquid medium supplement (insulin-transferrin-sodium selenite), 2 ng/mL bFGF and a cell culturing lipid additive (Sigma). The food material components used were egg white, soybean, wheat flour and bonito flakes (all in dry powder form). Each food component was dissolved at 0.1% with respect to the serum-free medium (0.02% for the bonito flakes), and the supernatant obtained after centrifugal separation was filtered with a 0.45 µm filter to remove the insoluble components, using the filtrate for the test. Myoblasts were seeded in a collagen-coated culture dish at approximately 5×10^3 cells/cm³, and cultured for 3 days. DMEM medium with addition of 10% FBS was used as a positive control. The fibroblasts were cultured for 3 to 4 days after seeding 1×10^4 cells/cm³ cells in a

tissue culture dish. DMEM medium with addition of 10% FBS was used as a positive control. After culturing in a CO₂ incubator set to 37°C, 5% CO₂ concentration, the viable cell count was determined by trypsin treatment and the percentage of the cell count when using each culture solution with respect to using serum-containing medium (i.e. the relative growth rate (%)) was calculated. The results are shown in Fig. 1. With culturing of bovine fibroblasts (A), the non-added negative control rate was 30%, but with addition of each food component, it increased to 59% (egg white), 48% (soybean), 40% (bonito flakes) and 37% (wheat flour), thus showing a particularly high cell proliferation rate when egg white was used. With culturing of bovine myoblasts (B), the non-added negative control rate was 20%, but with addition of each food component it increased to 34% (egg white), 24% (soybean) and 19% (wheat), thus showing a particularly high cell proliferation rate when egg white was used.

[0038]

(2) Chicken fibroblasts

A chicken fibroblast line (DF-1) obtained from ATCC was used to search for components that promote growth in serum-free medium. The serum-free medium used was DMEM medium with addition of 4 µM insulin, 1% penicillin-streptomycin (PS) solution, 0.2% BSA and 1% cell culturing lipid additive. The serum-containing medium used was DMEM medium with addition of 10% FBS, as a positive control. The food material components used were wheat flour, rice flour, corn flour, soybean, egg white and *Chlorella* (all dry powders). Each food component was dissolved to 0.5% in serum-free medium, the supernatant obtained after centrifugal separation was filtered with a 0.45 µm filter to remove the insoluble components, and the filtrate was diluted to 0.1, 0.02 and 0.01% with serum-free medium and used for testing.

About 6×10^4 cells/cm³ were seeded and cultured in a CO₂ incubator set to 37°C, 5% CO₂. After 3 days of culturing, the cells were stained with 0.5% crystal violet/20% methanol. The stained cells were eluted with 100% methanol and the absorbance at 590 nm was measured with a plate reader, calculating the absorbance when using each culture solution with respect to the absorbance when using the serum-containing medium. The food components were added at 0.1% to 0.01% for culturing of chicken fibroblasts, and the effect on cell proliferation was evaluated. The results are shown in Fig. 2(A). Cell growth equivalent to addition of serum was confirmed with soybean, *Chlorella* and egg white with 0.1% addition, but with 0.01% addition, cell growth activity was confirmed only with egg white. This suggested that the growth promoting effect of egg white may be higher even compared to the soybean and *Chlorella* which have been reported to be a substitute for serum in BMC Biotechnol (2023) Feb 8; 23(1):4 (NPL 7) and Prog Immunobiol Stand (1971) 5:202-8 (NPL 6). In Food Funct., (2020) 11, 2477-2488 (NPL 4), a slight growth promoting effect has been reported with egg white decomposition products, but here it was shown that the amount of egg white added can be reduced even more than disclosed

in that report. It was also demonstrated that proliferation is equivalent to that obtained in serum-containing medium, without using growth factors.

[0039]

(3) Chicken myoblasts

Chicken myoblasts were used to search for components that promote growth in serum-free medium. The serum-free medium used was DMEM medium with addition of 4 μM insulin, 1% penicillin-streptomycin (PS) solution, 0.2% BSA, 1% cell culturing lipid additive and 5 ng/mL bFGF. The serum-containing medium used was DMEM medium with addition of 10% FBS and 5 ng/mL bFGF, as a positive control. The food material components used were wheat flour, rice bran, chickpea, bonito flakes, fish gelatin, shrimp powder, egg white and pig gelatin (all dry powders). Each food component was dissolved to 0.1% in serum-free medium (0.025% for the egg white), the supernatant obtained after centrifugal separation was filtered with a 0.45 μm filter, and the filtrate was used for testing. The serum-containing medium was DMEM medium with addition of 10% FBS and 5 ng/mL bFGF, as a positive control. Myoblasts were seeded at approximately 2×10^4 cells/cm³ on a collagen-coated plate, and after culturing for 3 days, the viable cell count was determined by trypsin treatment and the percentage of the cell count when using each culture solution with respect to using serum-containing medium was calculated. The results are shown in Fig. 2(B). With chicken myoblasts as well, the growth promoting effect was confirmed to be highest with addition of egg white, being 73% of the proliferation when serum was added.

[0040]

Example 3: Comparison with serum-containing medium (pig fibroblasts)

The serum-free medium used was DMEM medium with addition of 4 μM insulin, 1% penicillin-streptomycin (PS) solution, 0.2% BSA and 1% cell culturing lipid additive. The serum-containing medium used was DMEM medium with addition of 10% FBS, as a positive control. After dissolving egg white as a food material component in serum-free medium at 0.025%, the centrifuged supernatant was filtered with a 0.45 μm filter to remove the insoluble components, and the filtrate was seeded at approximately 6×10^4 cells/cm³ and cultured for 3 days in a CO₂ incubator set to 37°C, 5% CO₂, after which a Cell Counting Kit solution (Doujin) was added. Color reaction was conducted for 2 hours in a CO₂ incubator, and the absorbance at 450 nm was measured. The ratio of the absorbance when using each culture solution with respect to the absorbance when using serum-containing medium was calculated. The results are shown in Fig. 3. When pig fibroblasts were cultured, no cell proliferation was observed with the non-added negative control (DMEM), but a high cell proliferation rate was obtained when using egg white as the food component.

[0041]

Example 4: Verification of effective concentration

A chicken fibroblast line (DF-1) and pig fibroblasts were used to search for components that promote growth in serum-free medium. The serum-free medium used was DMEM medium with addition of 4 μ M insulin, 1% penicillin-streptomycin (PS) solution, 0.2% BSA and 1% cell culturing lipid additive. The serum-containing medium used was DMEM medium with addition of 10% FBS, as a positive control.

For each food material component, egg white was dissolved to 0.2% in serum-free medium, the supernatant obtained after centrifugal separation was filtered with a 0.45 μ m filter to remove the insoluble components, and the filtrate was diluted with serum-free medium to 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125 and 0.0016%. Approximately 6×10^4 cells/cm³ were seeded and cultured for 3 days in a CO₂ incubator set to 37°C, 5% CO₂, after which a Cell Counting Kit solution (Doujin) was added. Color reaction was conducted for 2 hours in a CO₂ incubator, and the absorbance at 450 nm was measured. The ratio of the absorbance when using each culture solution with respect to the absorbance when using serum-containing medium was calculated. Pig and chicken fibroblasts at 0.003% or greater were confirmed to have proliferative activity of at least 70% of that with addition of serum. It was shown the amount of egg white added to pig and chicken cells can be reduced even more than disclosed in Food Funct. (2020) 11, 2477-2488 (NPL 4).

[0042]

Example 5: Analysis of molecular weight of dissolved egg white powder

The molecular weight distribution of an egg white solution was measured by gel filtration chromatography. For high-performance liquid chromatography (HPLC: product of Shimadzu Corp.), a Superdex 30 Increase 10/300 GL column (Cytiva) was mounted in a column oven set to 37°C, and the flow channel was equilibrated by flowing through phosphate buffer (pH = 7.4) containing 280 mM NaCl (hereinafter referred to as “eluent”) at a flow rate of 0.5 mL/min. Next, 50 μ L each of sample solution and standard solution were injected, the absorption wavelength at 214 nm was detected at each elution time, and the molecular weight distribution was confirmed by the obtained peak. The average molecular weight was calculated from a calibration curve drawn from the retention time with respect to the standard product molecular weight, using GPC analysis software (Shimadzu Corp.). The sample solution used was 0.2% egg white powder dissolved in water and passed through a 0.45 μ m filter, and diluted 10-fold with eluent. The standard solutions used were solutions containing 0.5 mg/mL each of ovalbumin, ovomucoid, cytochrome c, aprotinin and (Pro-Pro-Gly)₅. Since the peak for the egg white solution was detected with a shorter retention time than the retention time (27.7 minutes) for the peak for cytochrome c in the standard solution, the main component of the egg white solution was larger than cytochrome c (105 aa). Calculation with analysis software revealed an average molecular

weight of approximately 37,000 Da (corresponding to 336 aa). This was a larger molecular weight than reported in Food Funct. (2020) 11 and 2477-2488 (NPL 4) (≤ 15 aa).

CLAIMS

[Claim 1]

A cell proliferation medium containing basal medium and egg white or dried egg white as a cell growth promoting agent.

[Claim 2]

The medium according to claim 1, wherein the proliferated cells are cells to be used for producing cultured meat.

[Claim 3]

The medium according to claim 1, wherein the medium does not contain animal-derived serum.

[Claim 4]

The medium according to claim 3, wherein the animal-derived serum is fetal bovine serum (FBS).

[Claim 5]

The medium according to claim 1, wherein the cells include fibroblasts or muscle tissue cells.

[Claim 6]

The medium according to any one of claims 1 to 5, wherein the cells are cells derived from a cow, pig or chicken.

[Claim 7]

A method for preparing cells for producing cultured meat, the method comprising a step of culturing cells in medium containing basal medium and egg white or dried egg white as a cell growth promoting agent.

[Claim 8]

The method according to claim 7, wherein the medium does not contain animal-derived serum.

[Claim 9]

The method according to claim 8, wherein the animal-derived serum is fetal bovine serum (FBS).

[Claim 10]

5 The method according to claim 7, wherein the cells are derived from a cow, pig or chicken.

[Claim 11]

The method according to claim 10, wherein the cells include at least one type of cell selected from the group consisting of fibroblasts and muscle tissue cells.

10

[Claim 12]

A method for producing cultured meat, the method comprising a step of accumulating cells prepared by the method according to any one of claims 7 to 11.

15 [Claim 13]

The method according to claim 12, wherein the prepared cells are accumulated together with one or more substances selected from the group consisting of other cells, blood, tissue and extracellular matrix.

20 [Claim 14]

The method according to claim 13, wherein the other cells are cultured cells or cells acquired from an animal.

[Claim 15]

25 The method according to claim 12, which further comprises culturing after accumulation.

[Claim 16]

A cell growth promoting agent for producing cultured meat, which comprises egg white or dried egg white.

30

[Claim 17]

The cell growth promoting agent according to claim 16, which is added to animal-derived serum-free medium.

35 [Claim 18]

The cell growth promoting agent according to claim 16 or 17, which promotes growth of at

least one type of cell selected from the group consisting of fibroblasts and muscle tissue cells.

[Claim 19]

5 The cell growth promoting agent according to claim 18, wherein the cells are cells derived from a cow, pig or chicken.