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"Composition for Promoting Growth of Bifidobacteria and the Method for Manufacture Thereof".

This invention relates to a composition capable of promoting growth of bifidobacteria, and a method for manufacture thereof.

A number of substances capable of promoting growth of bifidobacteria (hereinafter referred to as Bifidus growth factors) have been reported, and there has been a variety of proposals for manufacturing methods. Powdered milk containing such substances are described for instance in the Japanese Patent Publications Nos. 32908/66, 6510/70, 6865/70, 21606/70, 40956/74 and 40957/74. However, most of the Bifidus growth factors reported so far have been found to be effective only through culture tests conducted outside of the living body, the activity of these factors inside the living body remaining thus unclear or often unsatisfactory.

Bifidobacteria form a genus of bacterium living in human intestines with well-known useful physiological significance. Needless to say, efforts have been made to raise the ratio of said bacteria relative to other bacteria living in the intestines of artificially fed infants. Aside from the case of using the Bifidus growth factors only for growth of bifidobacteria outside of a living body, Bifidus growth factors for use in food or culture media should desirably exhibit positive and sufficient effects within the living body as well.

The object of the present invention is to provide Bifidus

growth factors that may exhibit high activity in the living body.

Towards this object, the present invention provides a composition for promoting growth of bifidobacteria, the composition containing as effective constituent an oligosacchride having the general formula Gal-$(Gal)_n$-Glc, wherein Gal denotes a galactose residue, Glc a glucose residue and n an integer of 1 to 4.

The present invention also resides in a method for manufacture of a material for promoting growth of bifidobacteria characterized in that lactose or a lactose-containing material is treated with a β-galactosidase, usually that produced by Aspergillus oryzae.

Most of the oligosaccharides which are the effective constituents contained in the Bifidobacteria growth promoting composition or Bifidus growth factor of the invention have been discovered by the present inventors during their research into transfer reactions taking place along with hydrolysis when lactose is acted upon by β-galatosidase. Many reports have been made of the transfer reaction since the first one by Wallenfels appeared in 1951. The transferred oligosaccharides whose possible isolation has been reported are disaccharides, namely Gal-(β-1,2)-Glc, Gal-(β-1,3)-Glc, Gal-(β-1,6)-Glc, Gal-(β-1,3)-Gal and Gal-(β-1,6)-Gal and trisaccharides, namely, Gal-(β-1,6)-Gal-(β-1,4)-Glc and Gal-(β-1,6)-Gal-(β-1,6);Glc, wherein the mode of galactoside linkage is indicated within brackets. The presence of tetrasaccharides has been confirmed by Huber et al, but the structure thereof has not been clarified. Production of polysaccharides

including pentasaccharides has not been reported. The reports are limited to clarification of the transfer mechanism and studies of the structure of the oligosaccharides thus produced, and no report has been made of the relation between the transferred oligosaccharides and the growth of Bifidobacteria.

The oligosaccharides, which constitute the Bifidus growth factor and are of the above general formula (hereinafter simply referred to by the term "oligosaccaride") may be produced by treating lactose with β-galactosidase produced by Aspergillus oryzae. Following this treatment, there exist in the reaction mixture non-reacted lactose as well as galactose and glucose produced by hydrolysis. Therefore, in order to obtain a product having higher activity as Bifidus growth factors, the oligosaccharide concentration should be raised either by carrying out a suitable refining step following enzyme treatment, or by preselecting enzyme treatment conditions that will give a higher oligosaccharide ratio, or by a combination thereof.

Preferred methods of the present invention for manufacture of a material for promoting growth of Bifidobacteria will be explained in more detail, making reference to the drawings in which:

Figure 1 is a graph showing the relation between the saccharide yield and reaction time in the case of treating lactose with β-galactosidase;

Figure 2 is a graph showing the result of gel filtration on oligosaccharides; and

Figures 3 to 6 show the results of Examples 2 to 5 respectively.

Commercially available lactose may be used as it is for treatment by β-galactosidase, it being unnecessary to use a specially refined lactose. Whole milk or skim milk containing lactose as constituent may also be used as starting material.

For enzyme treatment, a substrate concentration of 10 to 50%; pH of 3 to 8; enzyme concentration of 1 to 100 units; and temperature of 20 to 50°C are preferred.

The reaction time has a marked effect on oligosaccharide yield. As seen from Figure 1 showing the relation between the reaction time and the amount of the resulting saccharide in a typical enzyme treatment, glucose, galactose and oligosaccharide increase in quantity substantially linearly at the initial stage of reaction but follow thereafter rather complicated curves, whereas the oligosaccharide will be reduced in quantity as from a certain time point. The time at which the oligosaccharide yield will be maximum may depend on other factors as well and hence the optimum reaction time should preferably be decided through experiments.

The oligosaccharide contained in the reaction mixture may be separated from other constituents by thin layer chromatography and determined by the Anthrone method.

The enzyme reaction may be terminated by heating the reaction mixture for 5 to 10 minutes at higher than 90°C.

The reaction mixture following an enzyme treatment may be used as Bifidus growth factors or one can use a composition such as dairy products containing same, either as it is or after suitable concentration or drying to a powdered form.

The reaction mixture may be subjected to purification as the occasion may demand for increasing the concentration of the effective constituent, namely, oligosaccharide. The purification may be carried out in a number of ways such as by preliminary refining of the reaction mixture with an ion exchange resin followed by passage through an activated charcoal column for adsorbing oligosaccharide thereto, and elution with aqueous alcoholic solutions. It is also possible to inoculate mono- and disaccharide fermenting micro-organisms into the reaction mixture for culture and consumption of the mono- and disaccharides, thereby facilitating isolation of oligosaccharides.

An analytic example of oligosaccharide contained in the inventive composition, namely that according to the Example 1 which is described below, and carried out under standard conditions is as follows:

a) molecular weight distribution

Figure 2 shows the result of gel filtration with Bio-Gel P-2. Peaks A, B and C correspond to tri-, tetra- and pentasaccharides, respectively, with the composition ratio as calculated from the area ratio of these peaks being about 55% for trisaccharide, about 32% for tetrasaccharide and about 13% for pentasaccharide and higher saccharides.

b) constituent saccharide

Lactose, oligosaccharide (not separated into its constituents) and tri- and tetrasaccharides of the oligosaccharide were hydrolyzed at 100°C for 4 hours in a 0.5 N-HCL solution. The same product was also hydrolyzed at 50°C for 4 hours with β-galactosidase.

The molar ratios of the resulting saccharides for these respective cases are illustrated in the following Table 1. It is seen from this Table that the molar ratios glucose/galactose for tri-, tetra- and pentasaccharides of the oligosaccharide amount to 1:2, 1:3 and 1:4, respectively.

TABLE 1

	galactose/glucose	
	acidic hydrolysis	enzyme decomposition
lactose	0.98	0.98
oligosaccharide	2.44	2.42
trisaccharide	1.94	1.92
tetrasaccharide	2.88	2.84
pentasaccharide	3.82	3.80

c) mode of linkage of constituent saccharides

From a product obtained by partial acid hydrolysis of main constituents of trisaccharide (as separated using active charcoal column chromatography), the presence of lactose and Gal-(β-1,6)-Gal was confirmed, in addition to glucose and galactose. From this result, and the glucose/galactose ratio in the wholly hydrolyzed product being 1:2, the trisaccharide structure was estimated to be Gal-(β-1,6)-Gal-(β-1,4)-Glc,

As a result of our similar analyses of a number of oligosaccharide constituents, it has been discovered that the oligosaccharide has the above general formula and that galactose-galactose linkage is β-1,3, β-1,4, or β-1,6 linkage, with the β-1,6 linkage being predominant and the galactose-glucose linkage

is β-1,3, β-1,4 or β-1,6 linkage with the β-1,4 linkage being predominant.

As discussed above, so far as isolated oligosaccharides of the inventive composition, are concerned, it may be affirmed that the individual oligosaccharides per se act as Bifidus growth factors, not to speak of a mixture of various oligosaccharides which will operate excellently for promoting the growth of bifidobacteria.

An advantage of the oligosaccharide contained in the inventive composition is that the action thereof is highly effective not only outside but also inside of the living body.

The growth promoting action on Bifidobacterium proper to the inventive composition has substantially no relation with the species of bifidobacteria and thus may be effective with Bifidobacterium breve, B. bacterium bifidum, B. infantis, B. adolescentis etc living fixed in human intestines.

The inventive composition may be reaction products obtained by treating highly purified oligosaccharide itself or any mixture containing oligosaccharide such as lactose or a lactose-containing material with β-galactosidase to oligosaccharide; powdered or fermented milk or the like food or medicine consisting of a secondary product of such reaction product or any desired food or medicine added with purified oligosaccharides

The present invention will be further described by referring to several examples.

Example 1

3.6 kg of lactose was dissolved in about 6 liters of warm water to which were added 50 ml of 1 M-acetic acid buffer solution (pH, 4.6), 100,000 units of β-galactosidase

and water to a 10 liter solution which was then reacted at 37°C for 5 hours. The reaction solution was then heated for denaturing enzyme, the denatured protein was filtered off and the remaining solution was passed through a column of anionic exchange resin and a column of cationic ion exchange resin. The filtrate was contacted overnight with a 30 x 30 cm column filled with active charcoal. The active charcoal was washed with 60 liters of deionated water for elution of monosaccharides. The active charcoal thus treated was then eluted with 60 liters of 5% ethanol and 60 liters of 50% ethanol in this order. The eluate obtained with the 50% ethanol was concentrated to about 7 liters, filtered in germ-free manner through a membrane filter (pore size, 0.2 μ) and again subjected to ion exchange to a transparent saccharide solution. The solution was concentrated in vacuum to a viscous solution of about 2.5 liters which again was filtered through a membrane filter and the filtrate solution was freeze dried to white oligosaccharide powders (hereafter stated as TOS).

Example 2

To 6 adult germ-free Fischer series female rats were administered 10 different bacteria (including B. breve) separated from human feces and these bacteria were made to be fixed in the intestines. Then, a) a 3% aqueous solution of lactulose, b) a 3% aqueous solution of TOS obtained from the Example 1 and c) a 3% aqueous solution of lactose were administered daily for 1 week periods in

the order of a) to c). The solutions were administered in amounts of 0.6 g per day as solid material, and 1 week rest periods were provided for changeover between the different solutions. During the interval, the rat feces were taken for measuring the number of live B. breve bacteria contained in the feces. The result is shown in Fig. 3.

As seen from this Figure, the number of B. breve bacteria is increased by about 10 times by administration of TOS. Only minor increase in the number of the bacteria may be noted by administration of lactose, whereas no change is caused for the case of lactulose administration.

Example 3

The following experiment was conducted with 6 adult common Fischer series male rats. The bifidobacteria used was B. breve or B. infantis which were obtained in such manner that bacteria resulting from 48-hour culture on VL-G medium and ensuing centrifugal separation were made to float in a milk culture medium and administered as it was. The administered amount was 2×10^8 per day in terms of the number of bacteria and 20 ml per day of 5% aqueous solution of TOS.

a) The rats were made to drink only the bacterial solution only one day.

b) The rats were administered every day for the second week only with bacterial solution.

c) The bacterial solution and TOS were administered every day for the third week.

d) Only TOS was administered every day for the fourth week.

Fig. 4 shows the result of the measured number of live bacteria of B bacteria (of the kinds as administered) contained in the rat's feces.

It may be seen from this Figure that the number of B bacteria in intestines is increased markedly by parallel administration of the bacterial solution and TOS and that the number of bacteria may be maintained upon interruption of the administration of the bacterial solution.

Example 4

The following experiment was conducted with 5 healthy adult men. The bacterial solutions as administered was that of B. breve obtained in the same way as in the Example 3.

Experiment schedule

first week administration only of bacterial solution

second week administration of bacterial solution and TOS (3 g per day)

third week administration of bacterial solution and TOS (10 g per day)

Number of administered bacteria: 10^9 per day

The way of administering TOS: Dissolved in lukewarm water to be drunk after lunch

Measurement: On the third, fifth and seventh days of the respective weeks, the numbers of the B. breve bacteria in the excrements of each man under test

were measured and averaged for the respective weeks.

The result of the experiments are as shown in Fig. 5, from which it is seen that the number of the B bacteria is increased markedly by TOS administration.

Example 5

The following experiment was conducted with 5 healthy adult men.

Experiment schedule	No TOS administration
first week	No TOS administration
second week	3 g per day of TOS
third week	10 g per day of TOS

The way of administering TOS: same as in Example 4

Measurement: The total number of bifidobacteria in excrements were measured in the same way as in Example 4.

The result of the experiment is shown in Fig. 6 from which it is seen that the number of bifidobacteria fixedly living in human intestines may be increased by TOS administration.

CLAIMS

1. A composition effective for promoting the growth of bifidobacteria, the composition containing as effective constituent at least one oligosaccharide represented by the general formula Gal-$(Gal)_n$-Glc, wherein Gal denotes a galactose residue, Glc a glucose residue and n an integer from 1 to 4, the oligosaccharide being obtained from lactose or a lactose-containing material by treatment with ß-galactosidase produced by Aspergillus oryzae.

2. A composition effective for promoting the growth of bifidobacteria, the composition being composed of oligosaccharides represented by the general formula Gal-$(Gal)_n$-Glc, wherein Gal denotes a galactose residue, Glc a glucose residue and n an integer of 1 to 4 and in which the galactose-galactose linkage is ß-1,3, ß-1,4 or ß-1,6 with the ß-1,6 linkage being predominant and the galactose-glucose linkage is ß-1,3, ß-1,4 or ß-1,6 with the ß-1,4 linkage being predominant, said oligosaccharides being produced by reacting a material containing 10-50% lactose with 1-100 units/ml of ß-galactosidase produced by Aspergillus oryzae at a temperature of 20-50°C and at a pH of 3-8, and optionally purifying the oligosaccharides with ion exchange resin, active charcoal and aqueous alcohol, or with microorganisms that ferment mono- and disaccharides.