

Method for preparing GOS having reduced allergenicity

Technical Field of the invention

The invention relates to the field of nutritional ingredients. More in particular, it relates to methods for producing hypoallergenic galacto- oligosaccharides and the use thereof in food and drink items. The invention particularly relates to the use of a beta-galactosidase derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) in the production of a hypoallergenic GOS preparation.

Background of the invention

The term “GOS” stands for galacto-oligosaccharides (GOS), which generally comprise a chain of galactose units and a terminal glucose unit, that arise through consecutive transgalactosylation reactions (transgalactosylation reactions), catalyzed by a beta-galactosidase. Some of the GOS components exist naturally in human breast milk and bovine colostrum. Typical GOS preparations mainly comprise di- to hexa-saccharides.

Various physiological functions of GOS have been reported, including the capacity to stimulate the growth of bifidogenic bacteria in the gut [1–3], to support normal gut transit [4], to contribute to natural defenses [5,6] and to enhance mineral absorption [7]. GOS has received particular attention for their prebiotic effects that promote the growth of *Bifidobacterium*, *Lactobacillus*, and other enteric bacteria. Therefore, GOS is commonly used in infant formula, beverages fermented by *Lactobacillus*, and yogurts. Some of these foods containing GOS are certified as Food for Specified Health Uses by the Consumer Affairs Agency in Japan, and GOS is certified as generally recognized as safe (GRAS) substances by the U.S. Food and Drug Administration (GRAS Notices: GRN 233, 236, 285, 286, 334, 484, 489, 495, 518, and 569).

In general, GOS is produced by a transglycosylation reaction (in particular a transgalactosylation reaction) with a beta-galactosidase enzyme (enzyme class EC.3.2.1.23). beta-Galactosidase enzymes are produced in many microorganisms such as *Bacillus circulans*, *Aspergillus oryzae*, *Kluyveromyces marxianus*, *Kluyveromyces fragilis*, *Sporobolomyces singularis*, and *Lactobacillus fermentum*. Beta-galactosidases differ in their three-dimensional structures, resulting in stereo- and regioselectivity of the glycosidic bonds that are formed. For example, typically a fungal beta-galactosidase derived from *Aspergillus* predominantly produces β 1-6 bonds (thus resulting in a GOS preparation that predominantly comprises β 1-6 bonds, which may be referred to as “6’-GOS”), while a bacterial beta-galactosidase derived

from *Bacillus* predominantly produce β 1-4 bonds (resulting in a GOS preparation that predominantly comprises β 1-4 bonds, which may also be referred to as “4’-GOS”). Moreover, beta-galactosidase produced by *B. circulans* possesses particularly strong transgalactosylation activity, and thus, GOS prepared by *B. circulans* is sold worldwide.

5 Since its introduction to the market (1999), approximately more than 100 million of infants have consumed infant formula containing GOS prepared by *B. circulans*. It has been proven to be a safe ingredient, with a GRAS status acknowledged by the FDA.

In the past few years, however, a small number of very rare cases of GOS-related allergy has been reported in South East Asia. Research has shown that certain oligosaccharide
10 structures present in GOS can exert an allergic response in very sensitive subjects [8].

Jyo *et al.* (Occup. Environ. Allergy 3, 12–20 (1992)) determined allergy symptoms after consumption of a lactobacillus beverage containing 6’-GOS produced by fungal beta-galactosidase. Allergy symptoms have also been observed with 4’-GOS produced by bacterial beta-galactosidase. In 2014, Kaneko *et al.* (Biosc. Biotechnol. Biochem. 78, 100-
15 108) observed that GOS produced by treating lactose with a beta-galactosidase derived from *B. circulans* may induce allergic reactions and revealed that the allergies were caused by two tetrasaccharides [Gal β 1-4 (Gal β 1-4 Gal β 1-6) Glc, Gal β 1-4 Gal β 1-4 Gal β 1-3 Glc]. These GOS allergy cases occurred in subjects who already had a history of atopy.

The present inventors aimed at the manufacture of a GOS preparation having
20 reduced allergenicity. In particular, they sought to identify a beta-galactosidase for use in the manufacture of a GOS preparation having a reduced capacity to induce an allergic response in a subject, as compared to a GOS preparation obtained by *Bacillus circulans* beta-galactosidase or by *Aspergillus oryzae* beta-galactosidase.

25 Summary

It was surprisingly observed that a GOS preparation produced by a β -galactosidase derived from a microorganism of the genus *Cryptococcus* has an unexpected low allergenicity. More specifically, it was found in an oral challenge test that GOS obtained using a *Cryptococcus terrestris* beta-galactosidase enzyme did not induce allergic symptoms
30 in human subjects suffering from hypersensitivity to GOS prepared by conventional *B. circulans* enzyme.

In one aspect, the present disclosure refers to the use of a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) having an amino acid sequence according to any of SEQ ID NO: 1, 2, 3, or 4, or an amino
35 acid sequence that is at least 95% identical to any of SEQ ID NO: 1, 2, 3, or 4, in

the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*. In another aspect, the present disclosure refers to a method for providing a hypoallergenic nutritional composition, comprising (i) contacting a lactose feed with a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* having an amino acid sequence according to any of SEQ ID NO: 1, 2, 3, or 4, or an amino acid sequence that is at least 95% identical to any of SEQ ID NO: 1, 2, 3, or 4, to provide a hypoallergenic galacto-oligosaccharide (GOS) preparation, and (ii) formulating said hypoallergenic GOS preparation together with at least one further hypoallergenic or non-allergenic ingredient into a hypoallergenic nutritional composition. In yet another aspect, the present disclosure refers to a hypoallergenic nutritional composition obtainable by a method according to the present disclosure.

Herewith, the disclosure provides the use of a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS)

preparation. Said hypoallergenic GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using beta-galactosidase derived from *Bacillus circulans* or *Aspergillus oryzae*. More in particular, the said hypoallergenic GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject having an increased chance to suffering from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using beta-galactosidase derived from *Bacillus circulans* or *Aspergillus oryzae*.

The disclosure also relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*), for use in at least partially preventing an (IgE-mediated) allergic response in a subject.

The disclosure also relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*), for use in at least partially preventing an (IgE-mediated) allergic response in a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*.

The disclosure further relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* for use in a nutritional composition for a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*.

The disclosure also provides a method for at least partially preventing hypersensitivity to a GOS preparation in a subject, comprising administering a (hypoallergenic) nutritional composition comprising a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) to the subject.

The disclosure further provides a nutritional composition comprising (i) a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* and (ii) at least one further ingredient selected from the group consisting of a hypoallergenic or non-allergenic protein source, preferably a protein hydrolysate, preferably a non-allergenic protein hydrolysate, free

amino acids, probiotics, LC-PUFA's and carbohydrates, such as lactose, saccharose, starch or maltodextrin.

Detailed description of the invention

5 The present invention relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or
10 from *Aspergillus oryzae*.

 It is to be noted that the organism "*Cryptococcus terrestris*" was recently renamed "*Papiliotrema terrestris*". The names "*Cryptococcus terrestris*" (*C. terrestris*) and "*Papiliotrema terrestris*" (*P. terrestris*) thus refer to the same organism. In the present
15 description and in the claims, the name *Cryptococcus terrestris* is used throughout. However, the *Cryptococcus terrestris* organism can also be referred to by the name *Papiliotrema terrestris*. *Cryptococcus terrestris* is the basionym of *Papiliotrema terrestris* (Liu et al., *Towards an integrated phylogenetic classification of the Tremellomycetes*, *Studies in Mycology* 81, 85-147 (2016)).

20 As used herein, the term "hypoallergenic GOS preparation" (abbreviated to HA-GOS) refers to a GOS composition which, when administered to a subject suffering from at least one type of GOS-related allergy, i.e. an allergy caused by GOS produced by *Bacillus circulans* beta-galactosidase and/or by GOS produced by *Aspergillus oryzae* beta-galactosidase, evokes a reduced allergic response when compared to a GOS preparation
25 produced by *Bacillus circulans* or *Aspergillus oryzae* beta-galactosidase.

 In one aspect, the term "hypoallergenic GOS preparation" refers to a GOS preparation that evokes at least a reduced allergenicity in a subject suffering from *Bacillus circulans*-derived GOS-related allergy. In this aspect, a hypoallergenic GOS preparation evokes
30 a reduced allergic response when compared to a GOS preparation produced by *Bacillus circulans*. More in particular, a hypoallergenic GOS preparation has a decreased score in a Skin Prick Test in the subject and/or in a Basophil Activation Test performed on a blood sample isolated from the subject when compared to a GOS preparation obtained by *Bacillus circulans*.

In another aspect, the term “hypoallergenic GOS preparation” refers to at least a reduced allergenicity in a subject suffering from *Aspergillus oryzae*-derived GOS-related allergy. In this aspect, a hypoallergenic GOS preparation evokes a reduced allergic response when compared to a GOS preparation produced by *Aspergillus oryzae*. More in particular, a hypoallergenic GOS preparation has a decreased score in a Skin Prick Test in the subject and/or in a Basophil Activation Test performed on a blood sample isolated from the subject when compared to a GOS preparation obtained by *Aspergillus oryzae*.

In one embodiment, the invention relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*.

In one embodiment, the invention relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject that is known to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*.

For example, the invention relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject that has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*.

In these embodiments, the subject is a mammal, in particular a human being. The subject may have any age. In a preferred embodiment, the subject is an adolescent or an adult. An adolescent is herein defined as a person having an age of from 13 to 20 years. An adult is herein defined as a person having an age of 20 years or higher. In another preferred

embodiment, the subject is a child having an age of 3 years (36 months) to 13 years. In yet another preferred embodiment the subject is child having an age of 0 to 3 years, preferably having an age of 18 months or above, more preferably having an age of 24 months or above. The rare GOS-related allergy has not been reported in subjects having an age of 18 months or below. Therefore, in a preferred embodiment, the subject is an adult, an adolescent, or a child, having an age of 18 months or above, preferably having an age of 24 months or above, more preferably having an age of 36 months or above.

In view of the localized incidence of the 4'-GOS and/or 6'-GOS-related allergies in South East Asia (e.g. Singapore, Japan), the subject is preferably of South East Asian origin.

The β -galactosidase enzyme(s) for use in the present invention for the manufacture of hypoallergenic GOS are known per se from patent application PCT/JP2016/089001 by Amano. PCT/JP2016/089001 claims a priority date of December 29, 2015, based on Japanese patent application no. 2015-257705.

PCT/JP2016/089001 discloses the screening of various kinds of microorganisms for β -galactosidase enzymes having desirable properties for industrial applications from viewpoints of heat resistance, pH stability, and others. This resulted in the finding of a microorganism (wild-type strain) of the genus *Cryptococcus*, in particular *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) which produces β -galactosidase that has a high optimum temperature and superior heat resistance, and in addition, excellent transgalactosylation activity. The *Cryptococcus terrestris*-derived beta-galactosidase described in PCT/JP2016/089001 are described in more detail below. PCT/JP2016/089001 also describes a method for producing oligosaccharides, comprising a step of subjecting the β -galactosidase enzyme to a reaction with a disaccharide, oligosaccharide, or polysaccharide having at least one of β -1,3-, β -1,4-, and β -1,6-linkages. Also disclosed is a method for producing oligosaccharides, comprising a step of subjecting the β -galactosidase enzyme to a reaction with lactose.

PCT/JP2016/089001 furthermore describes an oligosaccharide mixture obtained by the enzymes and methods disclosed therein, in particular an oligosaccharide mixture wherein 65% or more of the trisaccharides contained in the oligosaccharide mixture are composed of a linear oligosaccharide. It furthermore relates to the use of the β -galactosidase enzyme for the production of oligosaccharides, production of low-lactose milk, and productions of medicines or supplements for patients with lactose intolerance. PCT/JP2016/089001 specifically teaches that the GOS produced may be used as an intestinal *Bifidobacterium* growth factor.

Importantly however, PCT/JP2016/089001 does not address the allergenicity of the GOS mixture obtained using any of its enzymes. Hence, the present finding that GOS obtained by *Cryptococcus* enzymes according to PCT/JP2016/089001 has a surprisingly low allergenicity as compared to GOS prepared by *B. circulans* or by *A. oryzae* was not taught or suggested in the art.

The invention also relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*), for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject.

The present invention also provides a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris*, for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*. In one embodiment, the subject is known to suffer, or has an increased chance to suffer, from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans*. In another embodiment, the subject is known to suffer, or has an increased chance to suffer, from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Aspergillus oryzae*.

Also provided is a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris*, for use in a nutritional composition for a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae* beta-galactosidase. In one embodiment, the subject is known to suffer, or has an increased chance to suffer, from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans*. In another embodiment, the subject is known to suffer, or has an increased chance to suffer, from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Aspergillus oryzae*.

Also in these embodiments, the subject is a mammal, in particular a human being. The subject may have any age. In a preferred embodiment, the subject is an adolescent or an adult. In another preferred embodiment, the subject is a child having an age of 3 years (36 months) to 13 years. In yet another preferred embodiment the subject is child having an age of 0 to 3 years, preferably having an age of 18 months or above. In another preferred embodiment, the subject is an adult, an adolescent, or a child having an age of 18 months or above, preferably having an age of 24 months or above, more preferably having an age of 36 months or above. Also in these embodiments, the subject is preferably of South East Asian origin.

In another embodiment, the invention provides a method for at least partially preventing hypersensitivity to a GOS preparation in a subject, comprising administering a (preferably hypoallergenic) nutritional composition comprising a HA-GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* to the subject.

As used herein, a nutritional composition refers to any composition or formulation that goes into the alimentary canal for nutritional purposes, in whatever solid, liquid, gaseous state. Thus, a nutritional composition can be a food item or a drink item.

Also in this embodiment the subject is a mammal, in particular a human being, and may have any age. In a preferred embodiment, the subject is an adolescent or an adult. In another preferred embodiment, the subject is a child having an age of 3 years (36 months) to 13 years, preferably having an age of 18 months or above. In another preferred embodiment, the subject is an adult, an adolescent, or a child having an age of 18 months or above, preferably having an age of 24 months or above, more preferably having an age of 36 months or above. Also in these embodiments, the subject is preferably of South East Asian origin.

The invention also provides a nutritional composition comprising (i) a hypollergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* and (ii) at least one further ingredient selected from the group consisting of a hypoallergenic or non-allergenic protein source, preferably a non-allergenic milk protein hydrolysate, free amino acids, probiotics, a lipid source, and carbohydrates, such as lactose, saccharose, starch or maltodextrin.

Hypoallergenic or non-allergenic protein sources are known in the art, particularly for employment in infant formula. The terms non-allergenic hydrolysates and hydrolysates

substantially free of allergenic proteins as used herein are interchangeable. They refer to protein hydrolysates that can be administered to infants having intolerance against dietary proteins, more particularly cow's milk proteins, without inducing allergic reactions. In one embodiment, the at least one further hypoallergenic or non-allergenic ingredient is selected from non-
5 allergenic protein hydrolysates and hydrolysates substantially free of allergenic proteins, hypoallergenic protein sources, and hydrolyzed whey proteins. For example, US 5,039,532 discloses a hydrolyzed whey protein material from which allergens consisting of alpha-lactalbumin, beta-lactoglobulin, serum albumin and immunoglobulins have not been removed and wherein the hydrolyzed protein material including hydrolyzed allergens is in a form of
10 hydrolysis residues having a molecular weight not above 10,000 Da so that the hydrolyzed material is substantially free from allergenic proteins and allergens of protein origin. In one embodiment, a low-allergenic casein hydrolysate with peptides of maximally 3000 Da is included.

As a carbohydrate source, any type of carbohydrate, or a mixture of different
15 carbohydrates, can serve which is normally used in children's food formulations. Suitable carbohydrate sources are disaccharides such as lactose and saccharose, monosaccharides, such as glucose, and maltodextrins, starch and carbohydrate sources having a prebiotic effect. In one embodiment, human milk oligosaccharides are used.

The lipid source in a composition according to the invention may be any type of
20 lipid or combination of lipids which are suitable for use in (children's) nutritional products. Examples of suitable lipid sources are tri, di, and monoglycerides, phospholipids, sphingolipids, fatty acids, and esters or salts thereof. The lipids may have an animal, vegetable, microbial or synthetic origin. Of particular interest are polyunsaturated fatty acids (PUFAs) such as gamma linolenic acid (GLA), dihomogamma linolenic acid (DHGLA), arachidonic acid (AA),
25 stearidonic acid (SA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and conjugated linoleic acid (CLA). CLA is important in the protection against eczema and respiratory diseases in children. This particularly involves the cis-9, trans-11 and cis-12 isomers of CLA. Examples of suitable vegetable lipid sources include sun flower oil, high oleic sun flower oil, coconut oil, palm oil, palm kernel oil, soy bean oil,
30 etc. Examples of suitable lipid sources of animal origin include milkfat, for example anhydrous milkfat (AMF), cream, etc. In a preferred embodiment, a combination of milkfat and lipids of vegetable origin are used.

In an embodiment, the composition according to the invention comprises a probiotic. In the context of the present invention, the term "probiotic" refers to a strain of probiotic bacteria. Probiotic bacteria are known in the art. Suitably, the probiotic bacteria are not genetically modified. Suitable probiotic bacteria include bacteria of the genus
5 Bifidobacteria (e.g. *B. breve*, *B. longum*, *B. infantis*, *B. bifidum*), *Lactobacillus* (e.g. *L. Acidophilus*, *L. paracasei*, *L. johnsonii*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. casei*, *L. lactis*), and *Streptococcus* (e.g. *S. thermophilus*). *B. breve* and *B. longum* are especially suitable probiotics. Suitable *B. breve* strains may for example be isolated from the faeces of healthy human milk-fed infants.

10 The combination of a prebiotic and a probiotic is also referred to as a "synbiotic". The probiotic may be present in the composition at any suitable concentration, suitably in a therapeutically effective amount or "amount effective for treating" in the context of the invention. Suitably, the probiotic is included in the present composition in an amount of 10×10^2 - 10×10^{13} cfu per g dry weight of the composition, suitably 10×10^5 - 10×10^{12} cfu/g,
15 most suitably 10×10^7 - 10×10^{10} cfu/g.

Further, the composition may contain one or more conventional micro ingredients, such as vitamins, antioxidants, minerals, free amino acids, nucleotides, taurine, carnitine and polyamines. Examples of suitable antioxidants are BHT, ascorbyl palmitate, vitamin E, alpha and beta carotene, lutein, zeaxanthin, lycopene and phospholipids.

20 Further provided is a method for providing a hypoallergenic nutritional composition, comprising (i) contacting a lactose feed with a beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* to provide a hypoallergenic galacto-oligosaccharide (GOS) preparation, and (ii) formulating said hypoallergenic GOS preparation together with at least one
25 further hypoallergenic or non-allergenic ingredient into a hypoallergenic nutritional composition. Also provided is a hypoallergenic nutritional composition obtainable by a method of the invention.

The composition according to the invention can be used as a nutritional composition, nutritional therapy, nutritional support, as a medical food, as a food for special
30 medical purposes or as a nutritional supplement. The present composition is suitably an enteral composition. The composition is administered to, or intended to be administered to, a subject in need thereof. The subject is a mammal, in particular a human being, and the subject may have any age. In a preferred embodiment, the subject is an adult. The subject may e.g. be an

elderly person or a post-menopausal woman. The subject may also be a pregnant woman. Thus, in some embodiments, the present composition is a MUM composition for pregnant women. In another preferred embodiment, the subject is an adolescent.

In another preferred embodiment, the subject is a child having an age of 3 years (36 months) to 13 years. In this embodiment it is preferred that the child has an age of 3 years to 6 years. Thus, in some embodiments, the present composition is a growing-up milk for children having an age of 36 months or higher.

In yet another preferred embodiment the subject is child having an age of 0 to 3 years, preferably having an age of 18 months or above, more preferably having an age of 24 months or above. In this embodiment the subject is selected from the group consisting of children and infants, including toddlers, up to 3 years of age. Thus, in some embodiments, the present composition is an infant formula, a follow-on formula or a growing-up milk.

In a particular embodiment, the composition is for administration to subjects, in particular infants, at risk of developing allergy, especially cow's milk protein allergy (CMA). Infants that are known to be at risk of developing allergy include infants born from at least one parent who suffers from, or has suffered from, an atopic disorder (e.g. eczema) and/or an allergy, most in particular from CMA.

In view of the localized incidence of the 4'-GOS and/or 6'-GOS-related allergies in South East Asia (e.g. Singapore, Japan), the subject is preferably of South East Asian origin.

The enzyme for use according to the invention is a β -galactosidase derived from the yeast *Cryptococcus terrestris*. As described above, the organism *Cryptococcus terrestris* was recently renamed *Papiliotrema terrestris*, and the names "*Cryptococcus terrestris*" (*C. terrestris*) and "*Papiliotrema terrestris*" (*P. terrestris*) thus refer to the same organism. In the present description and in the claims, the name *Cryptococcus terrestris* is used throughout, but the *Cryptococcus terrestris* organism can also be referred to by the name *Papiliotrema terrestris*. Herein, by " β -galactosidase derived from *C. terrestris*" is meant a β -galactosidase enzyme produced by a microorganism (of either a wild-type strain or a mutant strain) which is classified into *Cryptococcus terrestris*, or a β -galactosidase enzyme obtained by genetic engineering procedures using the β -galactosidase gene from a microorganism (of either a wild-type strain or a mutant strain) which is classified into *Cryptococcus terrestris*. Therefore, the term " β -galactosidase derived from *Cryptococcus terrestris*" also encompasses a recombinant enzyme

that is produced by a host microorganism into which the β -galactosidase gene (or a modified gene thereof) obtained from *Cryptococcus terrestris* has been introduced.

In general, a β -galactosidase shows a lactose hydrolyzing activity (an activity to hydrolyze lactose by the action on the β -1,4 bond) and a transgalactosylation activity (an activity to transfer galactose). Therefore, the expression " β -galactosidase activity" as used herein is intended to include such two activities.

As demonstrated in the Examples section, the inventors of PCT/JP2016/089001 were successful in isolating and purifying β -galactosidase enzymes having the above-described properties, from *Cryptococcus terrestris* strain MM13-F2171 and its mutant strains M2 and M6. Mutant strains (M2 and M6) were obtained from *Cryptococcus terrestris* strain MM13-F2171 by means of mutagenesis with UV treatment. *Cryptococcus terrestris* strains MM13-F2171 and M2 have been deposited at a depository, as described below, and are readily available.

<*Cryptococcus terrestris* strain MM13-F2171>

Depository: Patent Microorganisms Depository, National Institute of Technology and Evaluation (Room 122, 2-5-8 Kazusa Kamatari, Kisarazu-shi, Chiba, 292-0818, JAPAN). Identification reference: *Cryptococcus terrestris* MM13-F2171. Date of deposit: December 10, 2015. Accession number: NITE BP-02177;

<*Cryptococcus terrestris* strain M2>

Depository: Patent Microorganisms Depository, National Institute of Technology and Evaluation (Room 122, 2-5-8 Kazusa Kamatari, Kisarazu-shi, Chiba, 292-0818, JAPAN). Identification reference: *Cryptococcus terrestris* APC-6431. Date of deposit: December 10, 2015. Accession number: NITE BP-02178

Accordingly, in one embodiment the enzyme used in the present invention is derived from *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178).

The present invention thus also relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS) preparation wherein said GOS preparation has a reduced capacity to induce an (IgE-

mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*, and wherein the beta-galactosidase is derived from *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178). Suitably, the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*. In one embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* and said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans*. In another embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Aspergillus oryzae* and said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Aspergillus oryzae*.

The invention further relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris*, for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject, wherein the beta-galactosidase is derived from *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178). The invention further relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris*, for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*, wherein the beta-galactosidase is derived from *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178). In one embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans*. In another embodiment the subject is known to suffer or has an increased

chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Aspergillus oryzae*,

Furthermore, the invention relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from

5 *Cryptococcus terrestris* for use in a nutritional composition for a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*, wherein the beta-galactosidase is derived from *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178). In one embodiment, the
10 subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans*, In another embodiment, the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Aspergillus oryzae*.

15 The invention also relates to a method for at least partially preventing hypersensitivity to a GOS preparation in a subject, comprising administering a (hypoallergenic) nutritional composition comprising a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* to the subject, wherein the beta-galactosidase is derived from *Cryptococcus terrestris* strain MM13-F2171
20 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178).

Also in these embodiments, the subject is a mammal, in particular a human being. The subject may have any age. In a preferred embodiment, the subject is an adolescent or an adult. In another preferred embodiment, the subject is a child having an age of 3 to 13 years. In yet another preferred embodiment the subject is child having an age of 0 to 3 years, preferably
25 having an age of 18 months or above. In another preferred embodiment, the subject is an adult, an adolescent, or a child having an age of 18 months or above, preferably having an age of 24 months or above. Also in these embodiments, the subject is preferably of South East Asian origin.

30 Furthermore, PCT/JP2016/089001 describes three kinds of β -galactosidase produced by mutant strains derived from the *Cryptococcus* microorganism (mutant strain enzymes 1, 2, and 3), and determined their amino acid sequences. These three β -galactosidase enzymes were found to have a partial sequence of the full-length amino acid sequence of the wild-type strain enzyme

(the wild-type strain enzyme is shown in Fig. 1A; SEQ ID NO: 1), which is deduced from its gene sequence. Specifically, these mutant enzymes are one having an amino acid sequence in which the N-terminal 130 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme are deleted, which is referred to as “mutant strain enzyme 1” for the purpose of description (see Fig. 1B; SEQ ID NO: 2); one having an amino acid sequence in which the N-terminal 136 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme are deleted (see Fig. 1C; SEQ ID NO:3), which is referred to as “mutant strain enzyme 2” for the purpose of description; and one having an amino acid sequence in which the N-terminal 141 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme are deleted (see Fig. 1D; SEQ ID NO:4), which is referred to as “mutant strain enzyme 3”.

Accordingly, a β -galactosidase enzyme derived from *C. terrestris* for use in a preferred embodiment of the present invention comprises the amino acid sequence of any one of SEQ ID NOs: 1, 2, 3 or 4, or an amino acid sequence equivalent to said amino acid sequence.

Also, a combination of two or more β -galactosidase enzymes derived from *C. terrestris* may be used, wherein each enzyme comprises an amino acid sequence of any one of SEQ ID NOs: 1, 2, 3 or 4 or an amino acid sequence equivalent to any one of said amino acid sequences. In one embodiment, at least one mutant enzyme (SEQ ID NO's 2, 3 or 4, or an equivalent thereof) is used. Preferably two or more mutant enzymes are used, optionally in combination with the wild type enzyme. For example, a combination of two or more enzymes, each enzyme comprising an amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, or an amino acid sequence equivalent to said amino acid sequences, is used. In a specific aspect, the enzyme combination comprises the three distinct mutant enzymes and the wildtype enzyme.

The term “equivalent amino acid sequence” in this case means an amino acid sequence which is partially different from the reference amino acid sequence (i.e. amino acid sequence of any one of SEQ ID NOs:1 to 4), but the difference does not substantially influence the function of the protein (beta-galactosidase activity). Thus, an enzyme having a polypeptide chain of the equivalent amino acid sequence shows a beta-galactosidase activity.

The degree of the activity is not particularly limited as long as the function of a beta-galactosidase can be exhibited, but is preferably equivalent to or higher than that of the enzyme

having a polypeptide chain of the reference sequence. Preferably, the length of the equivalent amino acid sequence is not longer than that of the sequence of SEQ ID NO: 1.

The term "partial difference in the amino acid sequence" typically means mutation (change) in the amino acid sequence caused by deletion or substitution of one to several (up to, for example, 3, 5, 7, or 10) amino acids composing the amino acid sequence, or addition, insertion, or a combination thereof of one to several (up to, for example, 3, 5, 7, or 10) amino acids. The difference in the amino acid sequence is acceptable as long as the beta-galactosidase activity is maintained (the activity may be varied to a degree). As long as the conditions are satisfied, the position of the difference in the amino acid sequence is not particularly limited, and the difference may arise in a plurality of positions. The term "plurality" means, for example, a number corresponding to less than about 20%, preferably less than about 15%, more preferably less than about 10%, even more preferably less than about 5% of the total amino acids, and most preferably less than about 1%. More specifically, the equivalent protein has, for example, about 80% or more, preferably about 85% or more, more preferably about 90% or more, much more preferably about 95% or more, even more preferably about 97% or more, and most preferably about 99% or more identity with the reference amino acid sequence.

The difference of the amino acid sequence may arise in a plurality of positions. Preferably, the equivalence protein is obtained by causing conservative amino acid substitution in an amino acid residue which is not essential for beta-galactosidase activity. The term "conservative amino acid substitution" means the substitution of an amino acid residue with another amino acid residue having a side chain with similar properties.

Amino acid residues are classified into several families according to their side chains, such as basic side chains (for example, lysine, arginine, and histidine), acidic side chains (for example, aspartic acid and glutamic acid), uncharged polar side chains (for example, glycine, asparagine, glutamine, serine, threonine, tyrosine, and cysteine), nonpolar side chains (for example, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan), β -branched side chains (for example, threonine, valine, and isoleucine), and aromatic side chains (for example, tyrosine, phenylalanine, tryptophan, and histidine). Conservative amino acid substitution is preferably the substitution between amino acid residues in one family.

The identity (%) between two amino acid sequences or two nucleic acid sequences (hereinafter, the term "two sequences" are used for representing either of two sequences) can be determined by the following procedure. Firstly, two sequences are aligned for optimum

comparison of the two sequences (for example, a gap may be introduced into the first sequence so as to optimize the alignment with respect to the second sequence). When a molecule (amino acid residue or nucleotide) at a specific position in the first sequence and a molecule in the corresponding position in the second sequence are the same as each other, the molecules in the positions are defined as being identical. The identity between two sequences is a function of the number of identical positions shared by the two sequences (i.e., identity (%) = number of identical positions / total number of positions %100). Preferably, the number and size of the gaps, which are required to optimize the alignment of the two sequences, are taken into consideration.

The comparison and determination of the identity between two sequences can be carried out by using a mathematical algorithm. A specific example of the mathematical algorithm that can be used for comparing the sequences includes an algorithm described in Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68 and modified by Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. However, the algorithm is not necessarily limited to this. Such an algorithm is incorporated in NBLAST program and XBLAST program (version 2.0) described in Altschul et al. (1990) J. Mol. Biol. 215: 403-10. In order to obtain an equivalent nucleic acid sequence, for example, BLAST nucleotide search with score = 100 and word length = 12 may be carried out by the NBLAST program. In order to obtain an equivalent amino acid sequence, for example, BLAST polypeptide search with score = 50 and word length = 3 may be carried out by the XBLAST program. In order to obtain gapped alignments for comparison, Gapped BLAST described in Altschul et al., (1997) Amino Acids Research 25(17): 3389-3402 can be utilized. In using BLAST and Gapped BLAST, the default parameters of the corresponding programs (e.g., XBLAST and NBLAST) can be used. In detail, see <http://www.ncbi.nlm.nih.gov>. Another example of the mathematical algorithm that can be used for comparing sequences includes an algorithm described in Meyers and Miller (1988) Comput. Appl. Biosci. 4: 11-17. Such programs are incorporated into the ALIGN program that can be used for, for example, GENESTREAM network server (IGH Montpellier, France) or ISREC server. When the ALIGN program is used for comparison of the amino acid sequences, for example, PAM120 weight residue table can be used in which a gap length penalty is 12 and a gap penalty is 4.

The identity between two amino acid sequences can be determined by using the GAP program in the GCG software package, using Blossom 62 matrix or PAM250 matrix with

the gap weight of 12, 10, 8, 6, or 4, and the gap length weight of 2, 3, or 4. The identity between two nucleic acid sequences can be determined by using the GAP program in the GCG software package (available at <http://www.gcg.com>), with the gap weight of 50, and the gap length weight of 3. The enzyme may be a portion of a larger protein (for example, a fusion protein).

5 Examples of the sequence added to a fused protein include the sequences / tags useful for purification of multiple histidine residues, and addition sequences which ensures stability in recombinant production.

The present invention also relates to the use of a beta-galactosidase (EC 3.2.1.23) in the
10 production of a hypoallergenic galacto-oligosaccharide (GOS) preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*, and wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4,
15 or an amino acid sequence that is at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 97%, and most preferably at least 99% identical to any of SEQ ID NO: 1, 2, 3 or 4.

The present invention also relates to the use of a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* in the production of a hypoallergenic galacto-oligosaccharide (GOS)
20 preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*, and wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4, or an amino acid sequence that is at least 80%,
25 preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 97%, and most preferably at least 99% identical to any of SEQ ID NO: 1, 2, 3 or 4. Suitably, the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*. In one embodiment the subject is known to suffer or
30 has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* and said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from

Bacillus circulans. In another embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Aspergillus oryzae* and said GOS preparation has a reduced capacity to induce an (IgE-mediated) allergic response in a subject, as compared to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Apergillus oryzae*.

The invention further relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23), for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject, wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4,

or an amino acid sequence that is at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 97%, and most preferably at least 99% identical to any of SEQ ID NO: 1, 2, 3 or 4. In a further embodiment, said subject is known to suffer from or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase

derived from *Bacillus circulans* or from *Aspergillus oryzae*. The invention further relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris*, for use in preventing, at least partially, an (IgE-mediated) allergic response in a subject known to suffer or having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by

transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or from *Aspergillus oryzae*, wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4, or an amino acid sequence that is at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 97%, and most preferably at least 99% identical to any of SEQ

ID NO: 1, 2, 3 or 4. In one embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans*. In another embodiment the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using a beta-galactosidase derived

from *Aspergillus oryzae*,

Furthermore, the invention relates to a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* for use in a nutritional composition for a subject known to suffer or

having an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or *Aspergillus oryzae*, wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4, or an amino acid sequence that is at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 97%, and most preferably at least 99% identical to any of SEQ ID NO: 1, 2, 3 or 4. In one embodiment, the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans*. In another embodiment, the subject is known to suffer or has an increased chance to suffer from hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Aspergillus oryzae*.

The invention also relates to a method for at least partially preventing hypersensitivity to a GOS preparation in a subject, comprising administering a (hypoallergenic) nutritional composition comprising a hypoallergenic GOS preparation obtainable by transgalactosylation of lactose using a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* to the subject, wherein the beta-galactosidase comprises an amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4, or an amino acid sequence that is at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, yet even more preferably at least 99%, and most preferably at least 98% identical to any of SEQ ID NO: 1, 2, 3 or 4.

Also in these embodiments, the subject is a mammal, in particular a human being. The subject may have any age. In a preferred embodiment, the subject is an adolescent or an adult. In another preferred embodiment, the subject is a child having an age of 3 to 13 years. In yet another preferred embodiment the subject is child having an age of 0 to 3 years, preferably having an age of 18 months or above. In another preferred embodiment, the subject is an adult, an adolescent, or a child having an age of 18 months or above, preferably having an age of 24 months or above. Also in these embodiments, the subject is preferably of South East Asian origin.

An enzyme for use in the present invention having the above-described amino acid sequence may also be prepared by a genetic engineering technique. For example, an appropriate host cell (for example, *Escherichia coli*) is transformed by a DNA encoding the present enzyme, and the protein expressed in the transformant is collected, and thereby preparing the present enzyme. The collected protein is treated as appropriate according to the intended use. The enzyme thus

obtained as a recombinant protein may be subjected to various modifications. For example, the enzyme composed of a recombinant protein linked to any peptide or protein can be obtained by producing a recombinant protein using a vector into which a DNA encoding the enzyme has been inserted together with other appropriate DNA. In addition, modification for causing
5 addition of a sugar chain and/or a lipid, or N- or C-terminal processing may be carried out. These modifications allow, for example, extraction of a recombinant protein, simplification of purification, or addition of biological functions.

The inventors of PCT/JP2016/089001 also characterized the enzymatic properties of the novel
10 β -galactosidase enzymes that had been obtained. Thus, the enzyme for use in the present invention may be characterized by enzymatic properties described below in (1) to (3), as described in PCT/JP2016/089001.

(1) Enzymatic action

15 The enzyme has a lactose hydrolyzing activity and a transgalactosylation activity, wherein the activity of the enzyme to transfer a galactosyl residue via β -1,4-linkage is superior to that via β -1,6-, β -1,3-, or β -1,2-linkage. That is, the enzyme has excellent activity to transfer a galactosyl residue via β -1,4-linkage. Therefore, the use of the enzyme allows an efficient production of a (hypoallergenic) product having the transferred sugar residue attached via β -1,4-linkage. For
20 example, reaction of the enzyme with lactose, which is a substrate for the enzyme, generates a mixture of trisaccharide oligosaccharides that is rich in linear oligosaccharides. In cases of trisaccharide oligosaccharides obtained when lactose is used as a substrate for reaction with the present enzyme under reaction conditions described in the Examples section of PCT/JP2016/089001 (in the subsection titled "Examination on oligosaccharide production
25 ability 1"), 65% or more, preferably 70% or more, further preferably 72% or more, still further preferably 73% or more, more preferably 75% or more, of the resulting trisaccharide oligosaccharides are composed of oligosaccharides with mere β -1,4-glycosidic linkages (O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose).

30 In these cases, the linear oligosaccharide produced by the enzyme is a galacto-oligosaccharide. In general, the galacto-oligosaccharide is represented by Gal-(Gal) $_n$ -Glc, wherein n is 1 to 5 or so, Gal is a galactose residue and Glc is a glucose residue. The type of linkage between sugar residues includes β -1,4, β -1,6, β -1,3, and β -1,2, and besides these, α -1,3, α -1,6, and others.

Therefore, a “galacto-oligosaccharide (GOS)” as used herein means a galacto-oligosaccharide of two or more sugar residues, i.e., having a degree of polymerization of 2 or more, excluding lactose.

5

(2) Optimum temperature

The enzyme has an optimum temperature of 70°C. Such a high optimum temperature of the enzyme is advantageous for use as an enzyme for the production of oligosaccharides. When the enzyme is used for the production of oligosaccharides, the process (reaction) temperature can be set to be higher. Herein, the optimum temperature can be determined by a method in which measurements are made using acetate buffer (pH 6.0) and with lactose as a substrate.

10

(3) Molecular weight

15

The wild-type strain enzyme and mutant strain enzymes 1 to 3 disclosed in PCT/JP2016/089001 each comprise a sugar chain(s); when the molecular weights of these enzymes were determined by SDS-PAGE after removal of N- and O-linked sugar chains, they were found to have a molecular weight of 104 kDa (for the wild-type strain enzyme), 64 kDa (for mutant strain enzyme 1), 61 kDa (for mutant strain enzyme 2), and 61 kDa (for mutant strain enzyme 3). On the basis of these findings, according to one embodiment, the enzyme without sugar chains for use in the present invention has a molecular weight of 104 kDa (by SDS-PAGE). According to another embodiment, the enzyme without sugar chains that is used in the present invention has a molecular weight of 64 kDa (by SDS-PAGE). According to further another embodiment, the enzyme without sugar chains has a molecular weight of 61 kDa (by SDS-PAGE). The above-mentioned enzymes when not subjected to treatments for removing sugar chains were found to have a molecular weight of 120 kDa (for the wild-type strain enzyme), 71 kDa (for mutant strain enzyme 1), 66 kDa (for mutant strain enzyme 2), and 66 kDa (for mutant strain enzyme 3).

20

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The enzyme for use according to the present invention may be further characterized by enzymatic properties described below in (4) to (6).

(4) Optimum pH

The enzyme has an optimum pH of 4 to 5. The optimum pH is determined, for example, on the basis of the results from measurements made using 0.1 M glycine buffer in the pH range of pH 2 to 3, 0.1 M citrate buffer in the pH range of pH 3 to 6, 0.1 M acetate buffer in the pH range of pH 5 to 6, 0.1 M phosphate buffer in the pH range of pH 7 to 8, and 0.1 M sodium carbonate buffer in the pH range of pH 9 to 10.

(5) pH stability

In one embodiment, the present enzyme exhibits stable enzymatic activity in the pH range of pH 2 to 8, and in another embodiment, in the pH range of pH 2 to 9. In other words, if the pH of an enzyme solution to be subjected to treatments is within this pH range, then the enzyme after pH treatments at 40°C for 30 minutes shows an activity of 80% or more of the maximal activity. The pH stability is determined, for example, on the basis of the results from measurements made using 0.1 M glycine buffer in the pH range of pH 2 to 3, 0.1 M citrate buffer in the pH range of pH 3 to 6,

0.1 M acetate buffer in the pH range of pH 5 to 6, 0.1 M phosphate buffer in the pH range of pH 7 to 8, and 0.1 M sodium carbonate buffer in the pH range of pH 9 to 10.

(6) Thermostability

In one embodiment, the enzyme for use as herein disclosed retains an activity of 80% or more of the maximal activity, even when the enzyme is treated for 30 minutes in acetate buffer (pH 6.0) under temperature conditions of 30°C to 60°C. In another embodiment, the enzyme retains an activity of 80% or more of the activity, even when the enzyme is treated for 30 minutes in acetate buffer (pH 6.0) under temperature conditions of 30°C to 65°C.

PCT/JP2016/089001 also discloses genes encoding an enzyme for use in the present invention.

In one embodiment, the gene includes a DNA that encodes an amino acid sequence of any one of SEQ ID NOs: 1 to 4. Specific examples of the embodiment are the cDNA consisting of the nucleic acid sequence of SEQ ID NO: 5 (encoding the amino acid sequence of SEQ ID NO: 1), the cDNA consisting of the nucleic acid sequence of SEQ ID NO: 6 (encoding the amino acid sequence of SEQ ID NO: 2), the cDNA consisting of the nucleic acid sequence of SEQ ID NO: 7 (encoding the amino acid sequence of SEQ ID NO: 3), and the cDNA consisting of the nucleic acid sequence of SEQ ID NO: 8 (encoding the amino acid sequence of SEQ ID NO: 4). A further example is the genome DNA consisting of SEQ ID NO: 16. This genome DNA corresponds to the cDNA of SEQ ID NO: 5.

The gene is typically used in preparation of the beta-galactosidase enzymes derived from *C. terrestris* disclosed in PCT/JP2016/089001 and used in the present invention. According to a genetic engineering procedure using the gene encoding the enzyme, the enzyme can be obtained in a more homogeneous state. Further, the method can be a preferable method
5 also in the case of preparing a large amount of the enzyme. Degeneracy of a codon is also considered.

As disclosed in PCT/JP2016/089001, the gene for use in the preparation of the beta-galactosidase enzymes derived from *C. terrestris* can be prepared in an isolated state by using
10 a standard genetic engineering technique, a molecular biological technique, a biochemical technique, a chemical synthesis, a PCR method (e.g. an overlap extension PCR) or a combination thereof, with reference to sequence information disclosed in the attached sequence listing.

In general, and as also described in PCT/JP2016/089001, when a part of DNA encoding a certain protein is modified, a protein encoded by the modified DNA may sometimes have the equal function to that of a protein encoded by the DNA before modification. That is to say, the modification of the DNA sequence does not have a substantial effect on the function of the encoded protein, so that the function of the encoded protein may be maintained before and after
15 the modification. Therefore the DNA encoding a protein may having a nucleic acid sequence equivalent to the reference base sequence (i.e., any one of SEQ ID NO: 5 to 8, 16) and having the β -galactosidase activity (hereinafter also referred to as “equivalent DNA”).
20

The “equivalent nucleic acid sequence” herein denotes a nucleic acid sequence which is partly different from the reference nucleic acid sequence but in which the function
25 (herein, β -galactosidase activity) of the protein encoded by the sequence is not substantially affected by the difference.

A specific example of the equivalent DNA includes DNA that hybridizes to the complementary base sequence of the reference nucleic acid sequence under stringent conditions. Herein, the “stringent conditions” are referred to as conditions in which a so-called
30 specific hybrid is formed but a nonspecific hybrid is not formed. Such stringent conditions are known to persons skilled in the art. Such stringent conditions can be set with reference to, for example, Molecular Cloning (Third Edition, Cold Spring Harbor Laboratory Press, New York) and Current protocols in molecular biology (edited by Frederick M. Ausubel et al., 1987). An

example of the stringent conditions can include a condition in which a hybridization solution (50% formamide, 10 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt solution, 1% SDS, 10% dextran sulfate, 10 µg/ml denatured salmon sperm DNA, and 50 mM phosphate buffer (pH 7.5)) is used and incubated at about 42°C to about 50°C, thereafter,
5 washed with 0.1 × SSC and 0.1% SDS at about 65°C to about 70°C. Further preferable stringent conditions can include, for example, a condition in which a hybridization solution (50% formamide, 5 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 × Denhardt solution, 1% SDS, 10% dextran sulfate, 10 µg/ml denatured salmon sperm DNA, and 50 mM phosphate buffer (pH 7.5)) is used.

10 As disclosed in PCT/JP2016/089001, another specific example of the equivalent DNA can include DNA encoding a protein having a nucleic acid sequence which includes substitution, deletion, insertion, addition or inversion in one or a plurality of nucleic acids (preferably one to several nucleic acids) in the reference sequence, and which has a β- galactosidase activity. The
15 substitution, deletion, or the like, of the nucleic acid may occur in a plurality of sites. The “plurality” herein denotes, for example, 2 to 40 bases, preferably 2 to 20 nucleic acids, and more preferably 2 to 10 nucleic acids, although it depends upon the positions or types of the amino acid residue in the three-dimensional structure of the protein encoded by the DNA. The equivalent DNA shows a 60% or more identity for example, preferably a 70% or more identity,
20 more preferably a 80% or more identity, more and more preferably a 85% or more identity, much more preferably a 90% or more identity, even more preferably 95% or more identity, and most preferably a 99 % or more identity with the reference nucleic acid sequence. The above-mentioned equivalent DNA can be obtained by modifying the reference DNA so as to include substitution, deletion, insertion, addition and/or inversion of nucleic acid by using treatment
25 with a restriction enzyme; treatment with exonuclease, DNA ligase, etc; introduction of mutation by a site-directed mutagenesis (Molecular Cloning, Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York) and random mutagenesis (Molecular Cloning, 20 Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York), and the like. Furthermore, the equivalent DNA can be also obtained by other methods such as irradiation
30 with ultraviolet ray. A further example of the equivalent DNA can include DNA having difference in nucleic acid as mentioned above due to polymorphism represented by SNP (single nucleotide polymorphism).

Therefore the nucleic acid may also be a nucleic acid having a complementary base sequence to the base sequence of the gene encoding the enzymes used in the present invention. For example, the nucleic acid may have a base sequence with an identity of at least about 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99%, or 99.9% to the base sequence of the gene encoding the enzymes used in the present invention, or the complementary base sequence thereto. The DNA may also be a recombinant DNA containing the gene encoding the enzymes used in the present invention.

The recombinant DNA is suitably provided in, for example, a form of a vector. The term “vector” in the present specification refers to a nucleic acid molecule that can transfer a nucleic acid inserted in the vector to a target such as a cell. A suitable vector is selected according to its intended use (cloning, expression of a protein) and in consideration of a kind of a host cell.

Examples include a M13 phage or an altered form thereof, a λ phage or an altered form thereof, and pBR322 or an altered form thereof (e.g., pB325, pAT153, pUC8), etc. as a vector having *Escherichia coli* as a host, pYepSec1, pMFa, and pYES2 as a vector having a yeast as a host, pAc, pVL, etc. as a vector having an insect cell as a host, and pCDM8, pMT2PC, etc. as a vector having a mammal cell as a host. The vector is preferably an expression vector. The “expression vector” refers to a vector capable of introducing a nucleic acid inserted in the expression vector into a target cell (host cell) and expressing it in the cell. The expression vector generally contains a promoter sequence necessary for expression of a nucleic acid inserted, an enhancer sequence for promoting expression, and the like.

An expression vector containing a selective marker can also be used. When such an expression vector is used, presence or absence (and its degree) of introduction of the expression vector can be confirmed using a selective marker.

Insertion of DNA into the vector, insertion of a selective marker gene (if necessary), insertion of a promoter (if necessary), and the like can be performed by using a standard recombinant DNA technique (for example, a known method of using a restriction enzyme and a DNA ligase, which can be referred in Molecular Cloning, Third Edition, 1.84, Cold Spring Harbor Laboratory Press, New York).

As described in PCT/JP2016/089001, an enzyme for use in the present invention is advantageously produced by a transformant into which the recombinant DNA encoding the beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* is introduced, such that the gene

exists as an exogenous molecule. Preferably, the transformant is prepared by transfection or transformation using the vector mentioned above. The transfection and transformation can be carried out using methods known in the art, for example, by a calcium phosphate co-precipitation method, electroporation, lipofection, microinjection, a method by Hanahan (Hanahan, D., J. Mol. Biol. 166, 557-580 (1983)), a lithium acetate method (Schiestl, R.H. et al., Curr. Genet. 16, 339-346 (1989)), protoplast - polyethylene glycol method (Yelton, M.M. et al., Proc Natl. Acad. Sci. 81, 1470-1474 (1984)), and the like.

The host cell is not particularly limited as long as the present enzyme can be expressed, and it can be selected from, for example, *Bacillus* genus bacteria (e.g. *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus circulans*, etc.), lactic acid bacteria (e.g. *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Bifidobacterium*, etc.), other bacteria (e.g. *Escherichia*, *Streptomyces*, etc.), yeast (e.g. *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torula*, *Torulopsis*, *Pichia*, *Schizosaccharomyces*, etc.), and filamentous fungi (Eumycetes) (e.g. *Aspergillus* genus fungi such as *Aspergillus oryzae* and *Aspergillus niger*, *Penicillium* genus fungi, *Trichoderma* genus fungi, *Fusarium* genus fungi, etc.).

PCT/JP2016/089001 also describes a method for producing a β -galactosidase enzyme which is advantageously used in the present invention. Disclosed is a method comprising the steps of culturing cells of *Cryptococcus terrestris* (step (1)); and a step of collecting the β -galactosidase enzyme from the cultured medium and/or cells (step (2)). Preferably, as the *Cryptococcus terrestris*, use is made of *Cryptococcus terrestris* strain MM13-F2171 or a mutant strain thereof, for example, *Cryptococcus terrestris* APC-6431 (strain M2) and further mutant strains thereof.

Conditions and methods for culturing cells of *Cryptococcus terrestris* are not particularly limited, as long as the enzyme is produced. Thus, methods and culture conditions that are suitable for culturing a microorganism to be used can be set as appropriate, with the proviso that the enzyme is produced. The cell culture may comprise mixtures of two or more *Cryptococcus terrestris* strains, for example wildtype strain MM13-F2171 and one or more mutant strains thereof, for example, *Cryptococcus terrestris* APC-6431 (strain M2) and/or *Cryptococcus terrestris* strain M6. In these cases, two or more enzymes can be isolated and purified simultaneously.

Although the culturing may be by either liquid culture or solid culture, liquid culture is preferably employed. As the medium, any medium can be used as long as microorganisms to be used can grow. For example, a medium supplemented with a carbon source such as glucose, sucrose, gentiobiose, soluble starch, glycerin, dextrin, molasses, and organic acid; and further, a nitrogen source such as ammonium sulfate, ammonium carbonate, ammonium phosphate, ammonium acetate, or peptone, yeast extract, corn steep liquor, casein hydrolysate, bran, and meat extract; and furthermore, an inorganic salt such as potassium salt, magnesium salt, sodium salt, phosphate salt, manganese salt, iron salt, and zinc salt, and the like, can be used.

In order to promote the growth of transformants to be used, vitamin, amino acid, and the like, may be added to the medium. The medium may be cultured under the aerobic conditions such that the pH of the medium is adjusted to, for example, about 3 to 8 (preferably about 4 to 7), and the culture temperature is generally about 20°C to 40°C (preferably about 25°C to 35°C) for 1 to 10 days (preferably 3 to 6 days). An example of the culture method may include a shake culture method, and an aerobic submerged culture method by using a jar fermenter.

After culturing under the above conditions, the target protein(s) is collected from the culture solution or the cell bodies (step (2)). When it is collected from the culture solution, the enzyme can be obtained by separation and purification by removing insoluble matters by, for example, filtration of culture supernatant, centrifugation, and the like, followed by carrying out, for example, concentration by ultrafiltration membrane, salting out by ammonium sulfate precipitation, dialysis, various types of chromatography of an ion-exchange resin or an appropriate combination thereof. On the other hand, when it is collected from cell bodies, the target protein(s) can be obtained by pulverizing the cell bodies by pressuring treatment, ultrasonic treatment, or the like, followed by separation and purification thereof similar to the above.

After collection of the cell bodies from a culture solution by filtration, centrifugation, etc., a series of processes (pulverizing, separation, and purification of cell bodies) mentioned above may be carried out. The β -galactosidase(s) for use in the present invention may also be produced by using the above-mentioned transformant. The transformant is then cultured under conditions such that a protein encoded by a gene introduced therein is produced (step (i)).

The culture conditions of transformant are known as to various vector-host systems, and a person skilled in the art can easily set an appropriate culture condition. Following the

culturing step, the produced protein (β -galactosidase) is collected (step (ii)). Collection and subsequent purification can be conducted in the same manner as the above embodiment. The purification degree of β -galactosidase(s) is not particularly limited. Furthermore, the final form of the β -galactosidase(s) may be a liquid state or a solid state (including a powder form).

5 The purified enzyme(s) can be provided in a powder form, for example, by freeze drying, vacuum drying, or spray drying. For example, the purified enzyme(s) may be previously dissolved in a phosphoric acid buffer solution, a triethanol amine buffer solution, a tris-hydrochloric acid buffer solution, or a GOOD buffer solution. Preferably, a phosphoric acid buffer solution and a triethanol amine buffer solution can be used. Note that, for the GOOD
10 buffer solution herein, PIPES, MES or MOPS is exemplified.

LEGENDS TO THE FIGURES

Figure 1: amino acid sequences of exemplary beta-galactosidase (EC 3.2.1.23) enzymes derived from *Cryptococcus terrestris*. A) SEQ ID NO: 1: wild-type enzyme; B) SEQ ID NO:
15 2: mutant strain enzyme 1; C) SEQ ID NO: 3: mutant strain enzyme 2; D) SEQ ID NO: 4: mutant strain enzyme 3.

Figure 2: Basophil activation in test subject #1 as measured by expression of the basophil activation marker CD203c (Figure 2a, MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 2b). Squares represent HA-GOS (solid
20 line in the graph). Diamonds represent REF-GOS (dotted line in the graph). For details see Example 2.

Figure 3: Basophil activation in test subject #2 as measured by expression of the basophil activation marker CD203c (Figure 3a, MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 3b). Squares represent HA-GOS (solid
25 line in the graph). Diamonds represent REF-GOS (dotted line in the graph). For details see Example 2.

Figure 4: Basophil activation in test subject #3 as measured by expression of the basophil activation marker CD203c (Figure 4a, MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 4b). Squares represent HA-GOS (solid
30 line in the graph). Diamonds represent REF-GOS (dotted line in the graph). For details see Example 2.

Figure 5: Basophil activation in test subject #4 as measured by expression of the basophil activation marker CD203c (Figure 5a, MFI = Mean Fluorescence) and percentage of cells

expressing the basophil activation marker CD63 (Figure 5b). Squares represent HA-GOS (solid line in the graph). Diamonds represent REF-GOS (dotted line in the graph). For details see Example 2.

Figure 6: Basophil activation in test subject #5 as measured by expression of the basophil activation marker CD203c (Figure 6a, MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 6b). Squares represent HA-GOS (solid line in the graph). Diamonds represent REF-GOS (dotted line in the graph). For details see Example 2.

EXPERIMENTAL SECTION

 β -Galactosidase derived from *C. terrestris*

The beta-galactosidase enzymes derived from *C. terrestris* as used in the present invention were
5 obtained from Amano Enzyme Inc. (Nagoya, Japan). These beta-galactosidase enzymes and
methods for their preparation are disclosed in PCT/JP2016/089001 by Amano. The method for
the preparation of beta-galactosidase enzymes derived from *C. terrestris*, as well as the enzyme
properties, as disclosed in PCT/JP2016/089001 are described below in paragraphs 1 to 8.

1. Obtaining a wild-type beta-galactosidase from *C. terrestris*

In order to obtain a β -galactosidase enzyme suitable for the production of galacto-
oligosaccharides, various kinds of microorganisms were screened. As a result, it turned out that
a microorganism of *Cryptococcus terrestris* contained in a soil sample that had been collected
near Heho Airport in Myanmar in October 2013 was a promising producer strain for β -
15 galactosidase. An attempt was made to purify β -galactosidase from this microbial strain
(*Cryptococcus terrestris* strain MM13-F2171). *Cryptococcus terrestris* strain MM13-F2171
was deposited on December 10, 2015 at the Patent Microorganisms Depository, National
Institute of Technology and Evaluation, under the name of *Cryptococcus terrestris* MM13-
F2171, to which the Accession Number NITE BP-02177 was assigned.

20 *Cryptococcus terrestris* strain MM13-F2171 was cultured in a liquid medium (2.0%
lactose, 2.0% Yeast Extract, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 5.0) at 30°C for 4 days
with shaking (at 200 revolutions per minute). After the culturing was completed, about 3 L
supernatant was collected by centrifugation, and then subjected to concentration and desalting
treatment with an ultra-filtration membrane (AIP-1013D with a membrane inner size of 0.8
25 mm; Asahi Kasei Chemicals Corp.) . In the desalting treatment, 20 mM acetate buffer (pH 6.0)
was used.

The concentrated solution was loaded onto an anion-exchange column HiTrap
DEAE FF (GE Healthcare Biosciences), which had been equilibrated with 20 mM acetate buffer
(pH 6.0). Absorbed fractions were eluted with a gradient using 20 mM acetate buffer (pH 6.0)
30 containing 1 M NaCl, and measured for enzyme activity. Fractions with enzyme activity were
pooled, and then subjected to dialysis against 20 mM acetate buffer (pH 6.0) containing 1.8 M
ammonium sulfate. The enzyme-active fraction obtained after the dialysis was loaded onto a
hydrophobic column HiTrap Phenyl HP (GE Healthcare Biosciences), which had been

equilibrated with 20 mM acetate buffer (pH 6.0) containing 1.8 M ammonium sulfate. Absorbed fractions were eluted with a gradient using 20 mM acetate buffer (pH 6.0), and measured for enzyme activity. Fractions with enzyme activity were pooled, and then subjected to dialysis against 20 mM acetate buffer (pH 6.0) containing 0.2 M NaCl.

5 The enzyme-active fraction obtained after the dialysis was loaded onto a gel filtration column HiLoad Superdex 200 prep grade (GE Healthcare Biosciences), which had been equilibrated with 20 mM acetate buffer (pH 6.0) containing 0.2 M NaCl, and then fractions with enzyme activity were collected. The enzyme had a molecular weight of about 266 kDa when determined by a gel filtration method using this HiLoad Superdex 200 prep grade column.

10 When this result is considered in combination with the results of SDS-PAGE analysis (see below), it is supposed that the enzyme is in the form of a dimer.

Subsequently, the molecular weight of the purified wild-type strain enzyme was determined by SDS-PAGE. First, samples of the purified wild-type strain enzyme were subjected to denaturation (in a denaturing buffer in a boiling water bath for 10 minutes), followed by
15 treatments for removal of *O*-linked sugar chains (using both *O*-glycosidase and neuraminidase; *O*-Glycosidase & Neuraminidase Bundle, New England Biolabs) and/or *N*-linked sugar chains (using PNGase F; New England Biolabs). The conditions for these enzyme treatments followed the protocols provided with the respective enzymes. After the treatments, the molecular weights of the resulting products were determined by SDS-PAGE.

20 The wild-type strain enzyme was found to have a molecular weight of 120 kDa after no treatment, 106 kDa after removal of *O*-linked sugar chains, 104 kDa after removal of *N*-linked sugar chains, and 104 kDa after removal of both *O*-linked and *N*-linked sugar chains.

2. Internal amino acid sequences of the purified enzyme

25 Analysis of the internal amino acid sequence of the purified enzyme revealed that the enzyme comprises the following internal amino acid sequences:

GVQYVDYNSPT (SEQ ID NO: 9)

FLFGWATAAQQ (SEQ ID NO: 10)

QAYQIGIFAEPIYNT (SEQ ID NO: 11)

30 PSIWDWAS (SEQ ID NO: 12), and

EEPPFAYVPE (SEQ ID NO: 13).

3. Determination of the gene sequence of the wild-type strain enzyme

An attempt was made to determine the gene sequence encoding the β -galactosidase produced by *Cryptococcus terrestris* strain MM13-F2171. *Cryptococcus terrestris* strain MM13-F2171 was cultured in a liquid medium (2.0% lactose, 2.0% Yeast Extract, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 5.0) at 30°C for 24 hours with shaking (at 200 revolutions per minute). After the culturing was completed, cells were harvested. Total RNA was prepared in accordance with the protocol of the RNeasy Mini Kit (QIAGEN) for RNA extraction from yeast cells (mechanical disruption of cells). The synthesis of cDNAs from the resulting total RNA was performed using the SMARTer RACE 5'/3' kit (TaKaRa), and then 5' and 3' RACE PCR reactions were carried out. The 5'RACE GSP primer used had the sequence GATTACGCCAAGCTTgcaagatcccgatctggtacgcctg (SEQ ID NO: 14), and the 3'RACE GSP primer used had the sequence GATTACGCCAAGCTTtctctgttgctggcgaccgcc (SEQ ID NO: 15). The base sequences of the resulting RACE PCR products were analyzed to determine the full-length cDNA sequence (SEQ ID NO: 5). The putative amino acid sequence encoded by the full-length cDNA sequence is of SEQ ID NO: 1.

By further investigation, the genomic DNA sequence encoding the β -galactosidase produced by *Cryptococcus terrestris* strain MM13-F2171 (SEQ ID NO: 16) was successfully determined.

4. Properties of the purified wild-type enzyme

(1) Optimum pH and pH stability

The optimum pH and pH stability of the purified wild-type enzyme were examined using a lactose hydrolyzing activity as an indicator. Examinations for optimum pH were performed using 0.1 M glycine buffer in the pH range of pH 2 to 3, 0.1 M citrate buffer in the pH range of pH 3 to 6, 0.1 M acetate buffer in the pH range of pH 5 to 6, 0.1 M phosphate buffer in the pH range of pH 7 to 8, and 0.1 M sodium carbonate buffer in the pH range of pH 9 to 10. The results from enzyme activity measurements at different pHs showed that the purified enzyme has an optimum pH of 4 to 5.

The pH stability of the purified enzyme was examined by heating it at 40°C for 30 minutes in buffers of different pHs (using the above-described buffers) and then measuring the residual enzyme activity. The results from residual enzyme activity measurements at different pHs showed that the purified enzyme exhibited stable enzyme activity in the pH range of pH 2 to 8.

(2) Optimum temperature and thermostability

To examine the optimum temperature of the purified enzyme, acetate buffer (pH 6.0) was used and the lactose hydrolyzing activity was measured at different temperatures. The results from enzyme activity measurements at different temperatures showed that the purified enzyme was found to have an optimum temperature of 70°C. To examine the thermostability of the purified enzyme, the lactose hydrolyzing activity was measured after the enzyme was heated in acetate buffer (pH 6.0) for 30 minutes at different temperatures. The results from enzyme activity measurements at different temperatures showed that the purified enzyme was stable between 30°C and 60°C and the enzyme activity was retained at levels of 80% or more of the activity.

5. Examination of oligosaccharide production ability of wild-type enzyme

(1) Methods

The purified enzyme was examined for the ability to produce oligosaccharides. One unit (1 U) of the purified wild-type strain enzyme per 1 g of lactose was added to aliquots of a 53% lactose solution that had been preheated to specified reaction temperatures, which then were subjected to reaction at those temperatures for 24 hours. The reaction solutions after the reaction was completed were analyzed by HPLC (under the conditions described below) to determine the composition of sugars contained therein. The results from determination of the composition of sugars allow an evaluation of the transglycosylation activity.

Examinations were made for the degrees of polymerization of galacto-oligosaccharides (GOSs) and of branching of trisaccharides when the production of galacto-oligosaccharides by the purified enzyme (wild-type strain enzyme) reached a yield of about 50%. Reactions were carried out in accordance with the above-described procedures, and at 65°C for 24 hours as conditions where the production of GOSs by the purified enzyme (derived from *Cryptococcus terrestris*) reached a yield of about 50%.

The degree of polymerization was determined by using an MCITM GEL CK04S column (Mitsubishi Chemical Corporation); H₂O as eluent; 0.4 ml/min flow rate; RI detector; 80°C column temperature.

The degree of branching was determined by using a Shodex column (registered trademark) Asahipak NH2P-40 3E (Showa Denko K.K.); MeCN : H₂O = 75 : 25 (vol : vol) as eluent; 0.35 ml/min flow rate; RI detector; 25°C column temperature.

(2) Results

Measurements results were used to calculate the content (%) of galacto-oligosaccharides (GOSs) in the total amount of sugars (total sugar), contained in the respective reaction solutions and the proportions (%) of respective GOSs with the indicated degrees of polymerization, at the indicated reaction temperatures. Results are shown in Table 1. The purified enzyme (wild-type strain enzyme) was found to have excellent GOS-producing ability. In addition, the wild-type strain enzyme was found to exhibit high levels of transglycosylation activity under high temperature conditions.

Table 1: GOS production with wild-type β -galactosidase at varying temperatures.

Enzyme	Reaction temperature	Amount of GOS	Ratio in GOS (%)		
			$\geq$ DP4	DP3	DP2*
WT strain enzyme	65°C	53.4	22.1	51.8	26.1
	70°C	54.2	25.8	47.4	26.8

* Lactose not included

Measurements results were used to calculate the proportions (%) of respective GOSs with the indicated degrees of polymerization. Typical results for the degrees of polymerization of GOSs when the purified enzyme (wild-type strain enzyme) was used are shown in Table 2. The wild-type strain enzyme (*Cryptococcus terrestris* derived enzyme) was found to have excellent GOS-producing ability and to efficiently produce oligosaccharides, particularly trisaccharides and higher saccharides.

Table 2: Comparison of GOS production with various enzymes.

Strain (enzyme)	Ratio in GOS (%)		
	$\geq$ DP4	DP3	DP2*
<i>Cryptococcus laurentii</i>	18.0	55.8	26.1
<i>Sporobolomyces singularis</i>	13.5	54.5	32.0
<i>C. terrestris</i> MM13-F2171 (WT strain enzyme)	16.7	57.5	25.8

* Lactose not included

Measurements results were used to calculate the proportions (%) of linear and branched oligosaccharide in the resultant trisaccharides and to compare the ratios of trisaccharides with

branched chain (i.e. the degrees of branching of trisaccharides) between the enzymes derived from *Cryptococcus terrestris* and known other β -galactosidase-producing strains. The results for the degrees of branching of GOSs when the purified enzyme (wild-type strain enzyme) was used are shown in Table 3. The wild-type strain enzyme (*Cryptococcus terrestris* derived enzyme) was found to produce predominantly linear oligosaccharides. Thus, it was revealed that the wild-type strain enzyme has transglycosylation activity in which the sugar chain is specifically transferred via β -1,4-glycosidic linkage and in particular, is less capable of transglycosylating so as to form β -1,6-glycosidic linkage.

Table 3: Comparison of Degree of branching of DP3 GOS produced with various enzymes.

Strain (enzyme)	Ratio in DP3 (%)		
	β 1-4	β 1-6	β 1-2, β 1-3
<i>Cryptococcus laurentii</i>	71.9	12.0	16.1
<i>Sporobolomyces singularis</i>	70.1	5.7	24.3
<i>C. terrestris</i> MM13-F2171 (WT strain enzyme)	76.3	1.5	22.1

6. Obtaining β -galactosidase enzymes produced by mutant strains, and determination of amino acid sequences and molecular weights thereof

Two mutant strains (M2 and M6) were obtained from *Cryptococcus terrestris* strain MM13-F2171 by means of mutagenesis with UV treatment. β -Galactosidase enzymes produced by these mutant strains were purified in procedures similar to those described above for the wild-type enzyme. Strains M2 and M6 each were found to have a high ability to produce mutant strain enzymes 1 to 3; strain M2 was observed to have a particularly high ability to produce mutant strain enzyme 1, and strain M6 to produce mutant strain enzymes 2 and 3. *Cryptococcus terrestris* strain M2 was deposited at December 10, 2015 at the Patent Microorganisms Depository, National Institute of Technology and Evaluation, under the name of *Cryptococcus terrestris* APC-6431, to which the Accession Number NITE BP-02178 was assigned.

The amino acid sequences of the obtained purified enzymes, i.e., one enzyme derived from mutant strain M2 (mutant strain enzyme 1) and two enzymes derived from mutant strain M6 (mutant strain enzymes 2 and 3), were determined. First, N-terminal amino acid sequences of mutant strain enzymes 1 to 3 were determined using a protein sequencer (PPSQ-31A, SHIMADZU CORPORATION). Then, the cDNA sequence of the wild-type strain enzyme (SEQ ID NO: 5) was searched for the base sequence corresponding to the N-terminal amino

acid sequence of each of the mutant strain enzymes, thereby to determine the cDNA sequence encoding each of the mutant strain enzymes. The amino acid sequence of mutant strain enzyme 1 (SEQ ID NO: 2) corresponds to one having a deletion of the N-terminal 130 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme (SEQ ID NO: 1), which is deduced from the cDNA sequence encoding the wild-type strain enzyme (SEQ ID NO: 5). Similarly, the amino acid sequence of mutant strain enzyme 2 (SEQ ID NO: 3) corresponds to one having a deletion of the N-terminal 136 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme (SEQ ID NO: 1), while the amino acid sequence of mutant strain enzyme 3 (SEQ ID NO: 4) corresponds to one having a deletion of the N-terminal 141 amino acid residues of the full-length amino acid sequence of the wild-type strain enzyme (SEQ ID NO: 1).

Subsequently, the molecular weights of these mutant strain enzymes were determined by SDS-PAGE. Procedures and conditions for removing sugar chains were in accordance with those described above for the wild-type enzyme. The molecular weights of the respective mutant strain enzymes were determined when the enzymes were subjected to no treatment, and treatments for removal of *N*-linked sugar chains, *O*-linked sugar chains, and both *N*-linked and *O*-linked sugar chains. On the basis of the results of SDS-PAGE analysis, it was observed that strain M2 produces mutant strain enzymes 2 and 3, in addition to mutant strain enzyme 1.

The mutant strain enzyme 1 was found to have a molecular weight of 71 kDa after no treatment, 65 kDa after removal of *O*-linked sugar chains, 64 kDa after removal of *N*-linked sugar chains, and 64 kDa after removal of both *O*-linked and *N*-linked sugar chains.

The mutant strain enzyme 2 was found to have a molecular weight of 66 kDa after no treatment, 63 kDa after removal of *O*-linked sugar chains, 61 kDa after removal of *N*-linked sugar chains, and 61 kDa after removal of both *O*-linked and *N*-linked sugar chains.

The mutant strain enzyme 3 was found to have a molecular weight of 66 kDa after no treatment, 62 kDa after removal of *O*-linked sugar chains, 61 kDa after removal of *N*-linked sugar chains, and 61 kDa after removal of both *O*-linked and *N*-linked sugar chains.

7. Examination of oligosaccharide production ability of mutant strain enzymes 1, 2 and 3

(1) Methods

To aliquots of a lactose solution was added the purified enzyme derived from strain M2 (mutant strain enzyme 1) or M6 (mutant strain enzyme 3), and the mixtures were subjected to reaction. Examinations were performed for the degrees of polymerization and branching of sugars

contained in the reaction solutions after the reaction was completed. The reaction conditions and measurements of the degrees of polymerization and branching were in accordance with those described above under 5.

5 (2) Results

Measurements results were used to calculate the content (%) of GOSs in the total amount of the sugars (total sugar) contained in the respective reaction solutions and the proportions (%) of respective GOSs with the indicated degrees of polymerization, at the indicated reaction temperatures (Table 4). The purified enzyme (mutant strain enzyme) was found to have excellent GOS-producing ability. In addition, the mutant strain enzyme was found to exhibit high levels of sugar transfer activity under high temperature conditions. It can also be found that there were no differences in GOS producing ability between the wild-type stain enzyme and the mutant strain enzyme.

15 Table 4: GOS production with mutant strain enzyme 3 at varying temperatures.

Enzyme	Reaction temperature	Amount of GOS	Ratio in GOS (%)		
			≥ DP4	DP3	DP2*
<i>C. terrestris</i> M6 (mutant strain enzyme 3)	50°C	46.9	6.4	68.9	24.7
	60°C	52.1	16.1	58.2	25.8
	65°C	53.2	20.9	53.4	26.0
	70°C	53.9	24.5	49.2	26.3

* Lactose not included

Measurements results were used to calculate the proportions (%) of respective GOSs with the indicated degrees of polymerization. Typical results for the degrees of polymerization of GOSs when the purified mutated enzymes derived from strains M2 (mutant strain enzyme 1) and M6 (mutant strain enzyme 3) were used are shown in Table 5. The mutant strain enzymes were found to have excellent GOS-producing ability and to efficiently produce oligosaccharides, particularly trisaccharides and higher saccharides. It can also be found that there were no differences in GOS producing ability between the wild-type stain enzyme and the mutant strain enzymes.

Table 5: Comparison of GOS production with various enzymes .

Strain (enzyme)	Ratio in GOS (%)		
	$\geq$ DP4	DP3	DP2*
<i>Cryptococcus laurentii</i>	18.0	55.8	26.1
<i>Sporobolomyces singularis</i>	13.5	54.5	32.0
<i>C. terrestris</i> M2 (mutant strain enzyme 1)	14.2	60.5	25.3
<i>C. terrestris</i> M6 (mutant strain enzyme 3)	19.9	54.2	25.8

* Lactose not included

Measurements results were used to calculate the proportions (%) of linear and branched oligosaccharide in the resultant trisaccharides and to compare the ratios of trisaccharides with branched chain (i.e. the degrees of branching of trisaccharides) between the wild-type strain enzymes and two mutant strain enzymes Table 6.

Table 6: Comparison of Degree of branching of DP3 GOS produced with various enzymes.

Strain (enzyme)	Ratio in DP3 (%)		
	β 1-4	β 1-6	β 1-2, β 1-3
<i>Cryptococcus laurentii</i>	71.9	12.0	16.1
<i>Sporobolomyces singularis</i>	70.1	5.7	24.3
<i>C. terrestris</i> M2 (mutant strain enzyme 1)	79.0	0.4	20.7
<i>C. terrestris</i> M6 (mutant strain enzyme 3)	75.2	2.7	22.1

The mutant strain enzymes (mutated, *Cryptococcus terrestris* derived enzymes) were found to produce predominantly linear oligosaccharides. Thus, it was revealed that the mutant strain enzymes have transglycosylation activity in which the sugar chain is specifically transferred via β -1,4-glycosidic linkage and in particular, is less capable of transglycosylating so as to form β -1,6-glycosidic linkage. It was also observed that the wild-type strain enzyme and the mutant strain enzymes have a comparable ability to produce GOSs and do not have any substantial differences in terms of properties as β -galactosidase. Mutant strain enzyme 2 is a β -

galactosidase enzyme of which the amino acid sequence is shorter by six amino acid residues at the N terminus than that of mutant strain enzyme 1 and longer by five amino acid residues at the N-terminus than that of mutant strain enzyme 3. Since it is apparent from the above results that an amino acid sequence in an N-terminal region does not affect enzymatic properties, it can be inferred that mutant strain enzyme 2 also have enzymatic properties equivalent to those of mutant strain enzymes 1 and 3.

8. Properties of mutant strain enzymes 1 and 3

The purified, mutated enzymes (see the above section described under 6.) were used to examine properties of mutant strain enzymes 1 and 3. The experimental methods were similar to those described for the wild-type strain enzyme (see the above section described under 4.).

(1) Optimum pH and pH stability

Mutant strain enzymes 1 and 3 each were found to have an optimum pH of 4 to 5. Mutant strain enzymes 1 and 3 each were found to exhibit stable activity in the pH range of pH 2 to 9.

(2) Optimum temperature and thermostability

Mutant strain enzymes 1 and 3 each were found to have an optimum temperature of 70°C. Mutant strain enzymes 1 and 3 each were found to be stable between 30°C and 65°C and to retain enzyme activity at levels of 80% or more of the activity.

EXAMPLE 1: Preparation of hypoallergenic GOS (HA-GOS).

Synthesis of GOS preparation

Four batches of a GOS preparation (referred to as PT 731-741-631-431) were produced by the transgalactosylation of lactose by four batches of *Cryptococcus terrestris* β -galactosidase enzyme (obtained from Amano Enzyme Inc., batch numbers GFEO0450731SDR, GFEO0450741SDR, GFEN1052631SDR and GFEO0750431SDR). For each batch, a lactose slurry (~50% (w/w) Lactopure, batch no. 502392; MFG:13-07-2014) was used as substrate. This lactose slurry was heated to 95°C to dissolve the lactose. Subsequently the lactose solution was cooled down to the reaction temperature of 65°C. A very mild citrate buffer (2-3mM) was added in order to adjust and stabilize the pH. Per gram lactose, 0.94 LU enzyme was used.

During the reaction the pH was adjusted by citric acid and sodium hydroxide when needed. 42 Hours after the enzyme addition, the reaction was stopped by heating 30 min. at 90 °C.

Down Stream Processing

- 5 After the enzyme reaction was terminated by heating, the solution was run on an Ion Exchange column (cation exchange followed by anion exchange)), followed by reduction of the pH to 3.2 using citric acid. After pasteurization at 75 °C the GOS was concentrated to approximately 75% dry matter.
- 10 The analyses of the thus obtained batches of GOS preparation are indicated in Table 7. In this table it is shown that the specifications of the four batches produced by applying four batches of *P. terrestris* β -Galactosidase enzyme are highly consistent (low inter-batch variation). Differences with REF-GOS, which is prepared by transgalactosylation of lactose under the action of *B. circulans*, are very small.

15

Table 7: Proximate analysis of the various batches of HA-GOS

Test Parameter	HA-GOS PT731	HA-GOS PT741	HA-GOS PT631	HA-GOS PT431	REF- GOS* (ref)
Dry matter (%)	76.08	75.09	76.39	76.79	74.26
Galacto- oligosaccharides (% on dry matter)	62.2	63.14	63.8	64.82	58.14
Nitrogen (% on dry matter)	0.0016	<0.0016	<0.0016	<0.0016	0.0016
Protein (% on dry matter)	0.01	<0.01	<0.01	<0.01	0.01
Sulphated ash (% on dry matter)	< 0.01	0.02	0.02	0.02	0.1
Lactose (% on dry matter)	18.3	17.6	16.62	17.07	20.04

Test Parameter	HA-GOS PT731	HA-GOS PT741	HA-GOS PT631	HA-GOS PT431	REF- GOS* (ref)
Glucose (% on dry matter)	18.4	18.16	18.39	17.24	20.36
Galactose (% on dry matter)	1.03	1.1	1.19	0.88	1.46
Nitrite	0.08	0.07	0.07	0.07	0.02
pH	3.34	3.24	3.08	3.2	2.9

* Conventional GOS prepared by transgalactosylation of lactose under the action of *B. circulans*.

GOS Degree of Polymerisation (DP) analysis was performed according to established methods.

- 5 Table 8 shows that the results of the Degree of Polymerization analysis of hypoallergenic GOS (HA- GOS) of the present invention are highly similar to those found in the reference GOS preparation obtained using the enzyme from *B. circulans* (REF-GOS).

Table 8: Analytical results for DP-analysis

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HA- GOS	Galactose	Glucose	DP2	DP3	DP4	DP5	DP6	DP7	Total
PT731	0.91	18.93	41.15	26.02	11.30	1.56	0.14	-	100
PT741	1.19	18.68	41.21	25.99	11.26	1.53	0.14	-	100
PT631	1.13	19.11	40.59	25.63	11.67	1.71	0.16	-	100
PT431	0.82	18.08	41.25	26.43	11.67	1.60	0.15	-	100
REF- GOS	1.31	21.12	37.42	22.02	10.76	4.88	1.90	0.60	100

EXAMPLE 2: Oral challenge test

This example describes a double-blind placebo-controlled oral challenge test to demonstrate the reduced allergenicity of HA-GOS in multiple human subjects with known galacto-oligosaccharide allergy. In addition, a skin prick test and basophil activation test were performed with HA-GOS.

Materials

HA-GOS (batch PT731; see Example 1) and a commercial GOS preparation obtained using *B. circulans* enzyme (REF-GOS) were included in the tests. The materials were stored at room temperature in the dark until use.

Subjects

Eligible subjects were selected from the cohort previously studied for the prevalence of GOS-allergy in a Singapore atopic population, as described in the paper by Soh et al., 2015¹⁰. Seven eligible subjects were approached for participation in the study. Two subjects refused to participate. Therefore, five adult subjects with confirmed GOS-related allergy were recalled to the clinic for a skin prick test, blood sample drawing and twice for a double-blind placebo-controlled oral challenge test with HA-GOS (minimum time between challenges was 2 weeks). The study was approved by the hospital's institutional ethical review board (IRB protocol "Testing hypoallergenicity of a modified galacto-oligosaccharide in patients with known galacto-oligosaccharide allergy". Written consent of all subjects was obtained prior to the start of the study.

Skin prick test, basophil activation test and oral challenge test

Skin prick testing to HA-GOS was carried out by the clinical research coordinator on the middle of the back or the forearm. Histamine and REF- GOS were used as positive controls. The wheal size for each sample was recorded and used as the degree of skin test reactivity.

A Basophil Activation Test was performed on patient blood samples. Heparinized peripheral blood aliquots (100 μ L) were pre-incubated at 37°C for 5 minutes and then incubated with 100 μ L of PBS (negative control), anti-IgE antibody (positive control, G7-18; BD Biosciences, San Jose, Calif) or diluted GOS samples for 15 minutes (37°C). After incubation, cells were washed

in PBS-EDTA (20 mmol/L) and then incubated with phycoerythrin-labeled anti-human IgE (Ige21; eBioscience, San Jose, Calif), biotin-labeled anti-human CD203c (NP4D6; BioLegend, San Jose, Calif), and fluorescein isothiocyanate-labeled anti-human CD63 (MEM-259, BioLegend) mAbs for 20 minutes at 48°C. Expression of CD203c and CD63 are both markers for basophil activation. After washing the cells with 1% BSA/PBS, allophycocyanin-conjugated streptavidin (BD Biosciences) was added and incubated for 15 minutes at 48°C. Thereafter, samples were subjected to erythrocyte lysis with 2 mL of FACS Lysing Solution (BD Biosciences). Cells were then washed, resuspended in 1% BSA/PBS, and analysed by means of FACSCalibur (BD Biosciences). Basophils were detected on the basis of side-scatter characteristics and expression of IgE (IgE^{high})⁷.

An Oral Food Challenge Test was performed by administration of escalating dosages of HA-GOS at 30 minute intervals to achieve a total cumulative dose of 4 grams (see Table 9 below). The total dose of 4 grams was chosen, as this was the maximum dose that triggered a clinical reaction in 5 patients who had anaphylaxis to REF-GOS, as reported by Chiang et al.⁷. A solution of 0.8 g GOS / 100 ml water was used in the study and prepared fresh on the day of the challenge.

Table 9: Dose regimen of the oral food challenge test

Time	Proportion of GOS mixture to meal size	Amount of GOS in grams	Amount of GOS in ml	Cumulative dose in grams
0'	5%	0.10	12.5	0.10
30'	25%	0.50	62.5	0.60
60'	70%	1.4	175	2.00
90'	100%	2.00	250	4.00

As placebo, we used a mixture of glucose, lactose and citric acid to mimic the non-oligosaccharide composition and taste of HA-GOS. Preparation of the test solution for the oral challenges was done by laboratory personnel not involved in the actual oral challenge testing. Furthermore, the preparation of the solution was checked by a second person to avoid any mix up. The physician performing the oral challenge test was blinded for the test material (HA-GOS or Placebo solution).

Results

Results skin prick test and oral challenge test

5 Seven subjects with prior proven REF-GOS-related allergy were eligible for an oral challenge with HA-GOS. These subjects had had reactions typical of an acute allergic reaction within 30 minutes of the threshold dose during challenge with REF-GOS. All had positive skin prick tests to REF-GOS.

10 Eventually, four subjects were challenged with HA-GOS and placebo in the current study (subject #1, #2, #4 and #5). Prior to the HA-GOS challenge, all were skin prick test negative to HA-GOS.

The detailed results of the oral challenge (OC) and skin prick test (SPT) with HA-GOS are presented in the table below. The response to a REF-GOS challenge (performed roughly two-
15 and-a-half years before the current challenge with HA-GOS) is also described in table 10, for reference.

Table 10: Results of the oral challenge (OC) and skin prick test (SPT) with HA-GOS

Subject number (male/ female)	Allergy back- ground	REF-GOS OC (date)	REF-GOS OC response	SPT REF- GOS (wheal)	SPT HA-GOS (wheal)	HA-GOS OC (date)	HA-GOS OC response
#1 (F)	AR	07-03-14	Sneeze, cough, chest tightness	3x4	0	21-11-16 C 12-12-16 P	none failed*
#2 (M)	AS	13-06-14	Wheeze, sneeze, chest tightness	5x5	0	09-12-16 P 19-12-16 C	none none
#3 (F)	AD, AR	15-08-14	Sneeze, cough, itchy eyes, chest tightness	3x3 no erythema	0	14-11-16 C	none

Subject number (male/female)	Allergy back-ground	REF-GOS OC (date)	REF-GOS OC response	SPT REF-GOS (wheal)	SPT HA-GOS (wheal)	HA-GOS OC (date)	HA-GOS OC response
#4 (F)	AR, AD	28-02-14	Chest tightness, cough, itchy throat	5x5	0	07-11-16 P 05-12-16 C	none none
#5 (F)	AR	04-04-14	Cough, sneeze, rash	5x5	0	31-10-16 P 11-11-16 C	none none

AR = allergic rhinitis, AS = asthma, AD = atopic dermatitis, OC = oral challenge, SPT = skin prick test, C = HA-GOS, P = placebo

*Subject #1 failed the placebo challenge test; she experienced an itch on the back, chest and tongue and felt giddy.

5

Subject #1 experienced symptoms of an allergic response (itch on the back, chest and tongue; giddy feeling) during the challenge with placebo, whereas she passed the oral challenge test with HA-GOS without any complaints. It was confirmed by medical practitioners that the subject had received placebo prior to experiencing these allergy symptoms. This is a known phenomenon that sometimes occurs during placebo-controlled challenges, and is probably caused by anxiety of the subject.

10

The data in Table 10 show that all four subjects which underwent a double-blind placebo-controlled challenge with HA-GOS showed no allergic symptoms upon consumption of HA-GOS.

15

Results basophil activation test

Figure 2-6 show the results of the basophil activation test performed on all five subjects prior to undergoing the oral challenge test.

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Figure 2 shows the results of the Basophil activation in test subject #1 as measured by expression of the basophil activation marker CD203c (Figure 2a, MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 2b). Squares represent HA-GOS (solid line in the graph). Diamonds represent REF-GOS (dotted line in the graph).

Similarly, Figures 3, 4, 5 and 6 show the Basophil activation in test subjects #2, #3, #4 and #5, respectively, as measured by expression of the basophil activation marker CD203c (Figures 3a, 4a, 5a and 6a; MFI = Mean Fluorescence) and percentage of cells expressing the basophil activation marker CD63 (Figure 3b, 4b, 5b and 6b). Squares represent HA-GOS (solid line in the graph). Diamonds represent REF-GOS (dotted line in the graph).

The results show that three subjects (#1, #3 and #4) had a negative basophil activation response to REF-GOS. This was also previously seen during other basophil activation tests with REF-GOS. These subjects, however, are confirmed allergic to REF-GOS, since they have a positive skin prick test and oral challenge test result in response to REF-GOS. The basophil response to HA-GOS was also negative in these subjects.

Subjects #2 and #5 showed a clear basophil activation response to REF-GOS, whereas HA-GOS did not result in basophil activation.

Conclusions

HA-GOS consumption showed no allergic responses during a placebo-controlled oral challenge test in n=4 REF-GOS-allergic subjects. Skin prick tests with HA-GOS were negative in all subjects tested. Also in the basophil activation test in REF-GOS allergic subjects, clearly reduced allergenicity was found with HA-GOS as compared to REF-GOS. Taken together, it can be concluded that HA-GOS is clearly hypoallergenic in comparison to REF-GOS.

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Claims

1. The use of a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) having an amino acid sequence according to any of
5 SEQ ID NO: 1, 2, 3, or 4, or an amino acid sequence that is at least 95% identical to any of
SEQ ID NO: 1, 2, 3, or 4, in the production of a hypoallergenic galacto-oligosaccharide (GOS)
preparation, wherein said GOS preparation has a reduced capacity to induce an (IgE-mediated)
allergic response in a subject known to suffer or having an increased chance to suffer from
hypersensitivity to a GOS preparation obtained by transgalactosylation of lactose using *Bacillus*
10 *circulans* or *Aspergillus oryzae*, as compared to a GOS preparation obtained by
transgalactosylation of lactose using a beta-galactosidase derived from *Bacillus circulans* or
from *Aspergillus oryzae*.
2. The use according to claim 1, wherein the beta-galactosidase is derived from
15 *Cryptococcus terrestris* strain MM13-F2171 (Accession Number: NITE BP-02177) or APC-
6431 (Accession Number: NITE BP-02178).
3. The use according to claim 1 or 2, wherein the beta-galactosidase comprises an
amino acid sequence according to any of SEQ ID NO: 1, 2, 3 or 4, or an amino acid sequence
20 that is at least 97%, preferably at least 98%, and most preferably at least 99% identical to any
of SEQ ID NO: 1, 2, 3 or 4.
4. The use according to any of the preceding claims, wherein said hypoallergenic GOS
preparation has a decreased score in a Skin Prick Test in the subject and/or in a Basophil
25 Activation Test performed on a blood sample isolated from the subject when compared to a
GOS preparation obtained by transgalactosylation of lactose using *Bacillus circulans* or
Aspergillus oryzae beta-galactosidase.
5. The use according to any of the preceding claims, wherein the subject is of South
30 East Asian origin.
6. The use according to any of the preceding claims, wherein the subject is an adult,
an adolescent, or a child, preferably having an age of 18 months or above.

7. A method for providing a hypoallergenic nutritional composition, comprising (i) contacting a lactose feed with a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* having an amino acid sequence according to any of SEQ ID NO: 1, 2, 3, or 4, or an amino acid sequence that is at least 95% identical to any of SEQ ID NO: 1, 2, 3, or 4, to provide
5 a hypoallergenic galacto-oligosaccharide (GOS) preparation, and (ii) formulating said hypoallergenic GOS preparation together with at least one further hypoallergenic or non-allergenic ingredient into a hypoallergenic nutritional composition.
8. Method according to claim 7, wherein said at least one further hypoallergenic or
10 non-allergenic ingredient is selected from non-allergenic protein hydrolysates, hydrolysates substantially free of allergenic proteins and hypoallergenic protein sources.
9. The method according to claim 7 or claim 8, wherein the nutritional composition is a composition for pregnant women, a growing up milk, a follow-up formula or an infant
15 formula.
10. The method according to claim 7 or claim 8, wherein the beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* (recently renamed *Papiliotrema terrestris*) is a beta-galactosidase (EC 3.2.1.23) derived from *Cryptococcus terrestris* strain MM13-F2171
20 (Accession Number: NITE BP-02177) or APC-6431 (Accession Number: NITE BP-02178).
11. The method according to claim 7 or claim 8, wherein the beta-galactosidase (EC 3.2.1.23) derived from *C. terrestris* comprises an amino acid sequence according to any one of SEQ ID NOs: 1 to 4 or an amino acid sequence that is at least 97%, preferably at least 98%,
25 and most preferably at least 99% identical to any one of SEQ ID NOs: 1 to 4.