

SPECIFICATION

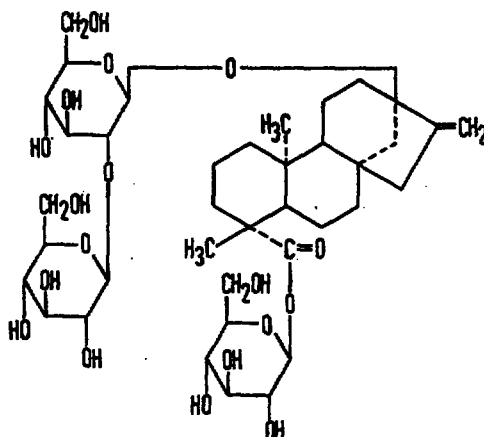
Alpha-glycosyl stevioside sweeteners

The present invention relates to a sweetener composition and to a process for its preparation.

Recently, because the use of artificial sweeteners such as dulcin, sodium cyclamate and saccharin has been banned or restricted as a result of the harmful side-effects which they are believed to have, there has been an ever-increasing demand for harmless natural sweeteners. In response to such demands, farmers and manufacturers of sweeteners have had to produce increasing amounts of stevioside.

Stevioside is a naturally-occurring glycoside obtained by extracting *Stevia rebaudiana* BERTONI (hereinafter referred to as *Stevia*), a member of the Chrysanthemum family. The part of the plant which grows above the ground, e.g. the leaves and stems thereof, are extracted in known manner. Stevioside, as shown in the formula below is a beta-glucosyl glycoside wherein the aglycon is steviol:

Chemical Structure of Stevioside



It is known to use stevioside, extracted from *Stevia* leaves, in crude or refined form or in mixtures with other sweeteners, as a sweetener to sweeten foods and drinks.

As is well known, the uses of stevioside or conventional stevioside-containing sweeteners are restricted and require special precautions or lead to certain problems as a result of a number of inherent disadvantages and properties.

Thus, for example, besides being sweet, stevioside imparts bitterness and astringency. Moreover, the sweetness of stevioside takes longer to take effect in a consumer's mouth than that of sucrose and gives a lingering, unpleasant aftertaste. Finally, stevioside is not readily soluble in water, its solubility being only 0.12% at 20°C.

According to the present invention, there is provided alpha-glycosyl stevioside, or a composition comprising an alpha-glycosyl stevioside, each for use as a sweetener, which sweetener is preferably obtained by subjecting an aqueous solution comprising stevioside and at least one alpha-glucosyl sugar compound to the action of an alpha-glucosyl-transferase.

According to a further aspect of the present invention there is provided a process for the preparation of a sweetener comprising an alpha-glycosyl stevioside, which process comprises subjecting an aqueous solution comprising stevioside and one or more alpha-glucosyl sugar compounds, preferably selected from maltooligosaccharides such as maltose, maltotriose, maltotetraose, partial hydrolyzates of starch, and sugar compounds containing an alpha-glucosyl residue such as sucrose, to the action of at least one alpha-glucosyltransferase which is capable of transferring an alpha-glucosyl residue to the stevioside to produce, as the sweetener, alpha-glycosyl stevioside or an alpha-glycosyl stevioside containing composition.

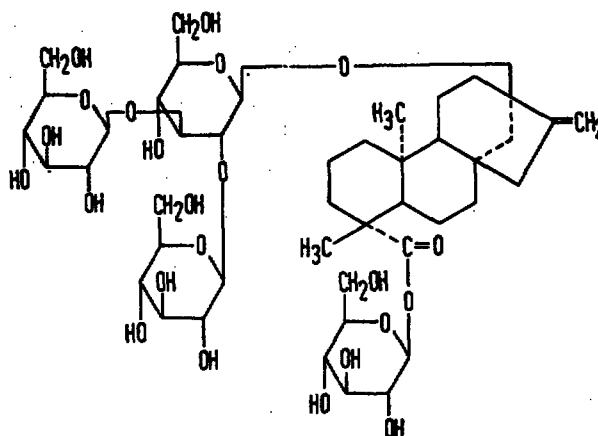
Examples of suitable such alpha-glucosyltransferases are alpha-glucosidase (E.C.3.2.1.20), alpha-amylase (E.C.3.2.1.1.), cyclodextrin glycanotransferase (E.C.2.4.1.19), and dextran sucrose (E.C.2.4.1.5).

The process of the present invention yields a sweetener composition containing the alpha-glycosyl stevioside formed by the reaction, which composition possesses the following outstanding desirable properties and features that are unattainable with conventional stevioside products or stevioside containing sweetener compositions. More particularly, the new sweetener possesses neither bitterness nor astringency, and imparts a mild, soft, round and pleasant sweetness, without a lingering unpleasant aftertaste, and possesses a substantially and desirably improved water solubility.

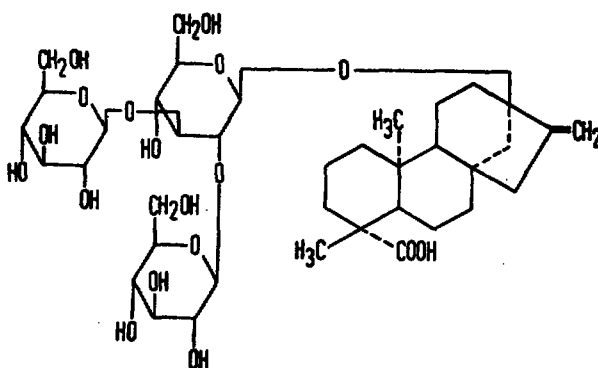
In addition, it has been ascertained by thin-layer chromatography that the process of the invention converts a portion or nearly all of the stevioside used in the reaction into an alpha-glycosyl stevioside, such as alpha-monoglucosyl stevioside, alpha-diglucosyl stevioside and alpha-triglucosyl stevioside.

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Chemical Structure of Rebaudioside B



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starch hydrolyzate (maltodextrin) with a D.E. of about 30 are suitable as substrates for alpha-amylase (E.C.3.2.1.1); a cyclodextrin or alpha-glucosyl sugar compounds ranging from a gelatinized starch product with a D.E. not higher than 1 to a partial starch hydrolyzate with a D.E. of about 60 are suitable as substrates for cyclodextrin glucanotransferase (E.C.2.4.1.19); and sucrose is suitable as a substrate for dextranucrase (E.C.2.4.1.5).

The above-mentioned gelatinized starch product or partial starch hydrolyzate may be a product obtained from cereal starch, e.g., wheat starch and corn starch, tuber- and root-starch, e.g., sweet potato starch, potato starch and tapioca starch.

Such a gelatinized starch product is prepared by gelatinization achieved by heating a starch slurry to a temperature above the gelatinization point, generally from 70°C to 140°C. The partial starch hydrolyzate is obtained by hydrolyzing a starch slurry with acid(s) and/or amylase(s) to the desired D.E.

In the process of the present invention, the alpha-glucosyl sugar compound may be of one type, or alternatively more than one type of alpha-glucosyl sugar compound may be used concurrently.

Any alpha-glucosyltransferase may be used in the process of the present invention so long as it forms alpha-glycosyl stevioside without decomposing stevioside when it is allowed to act on an aqueous solution comprising an alpha-glucosyl sugar compound (a suitable substrate for the enzyme) and stevioside.

Examples of suitable alpha-glucosyltransferases are alpha-glucosidase (E.C.3.2.1.20) derived from animal sources such as pig liver, plant sources such as buck wheat seed, fungi such as those of genera *Mucor* and *Penicillium*, and yeasts such as those of genus *Saccharomyces*; alpha-amylase (E.C.3.2.1.1) derived from various microorganisms, especially bacteria of genus *Bacillus*, and from fungi of genus *Aspergillus*; cyclodextrin glucanotransferase (E.C.2.4.1.19) derived from various microorganisms, especially bacteria of genus *Bacillus*, and genus *Klebsiella*; dextran sucrose (E.C.2.4.1.5.) derived from bacteria of genus *Leuconostoc*; dextrin dextranase (E.C.2.4.1.2) derived from bacteria of genus *Acetobacter*; and amylosucrase (E.C.2.4.1.4) derived from bacteria of genus *Neisseria*.

Purification of the alpha-glucosyltransferase is not necessarily required so long as the above described conditions are satisfied and usually the process of the invention can be performed with a crude alpha-glucosyltransferase.

For example, a crude alpha-glucosyltransferase of animal or plant origin is obtained by salting-out using ammonium sulphate an extraction solution of ground or minced animal or plant tissues, or by precipitating and separating the extraction solution with organic precipitants such as alcohol or acetone. If necessary, the crude transferase may be purified by any known method before use.

Known microbial alpha-glucosyltransferase include those derived from bacteria, fungi, yeasts and other microorganisms. Usually, the solid culture method used for "koji" and the liquid culture method such as the tank culture method are used for the production of such transferases. Similarly as in the case of transferases of animal or plant origin, microbial alpha-glucosyl-transferase is prepared by extraction and, if necessary, may be purified by any known method prior to use.

Although in the case of liquid culture the whole culture broth may be used as the alpha-glucosyltransferase, usually the supernatant from which insolubles have been removed is used as the alpha-glucosyltransferase. Alternatively, the elaborated enzyme may be utilized in the form of cells or after extracting the enzyme from the cells and, if necessary, may be purified further prior to its use.

Commercially available alpha-glucosyltransferase is also suitable for use in the present process.

The alpha-glucosyltransferase can be immobilized on suitable supports and such immobilized enzyme may be used repeatedly in a batchwise process or in a continuous process. The sweetener composition of the invention can be prepared by cultivating an alpha-glucosyltransferase-producing microorganism, animal tissue or plant tissue in a medium containing an alpha-glucosyl sugar compound and stevioside thereby to form the desired alpha-glycosyl stevioside containing composition.

The reaction conditions used in the process of the present invention are such that the alpha-glucosyltransferase can act on the aqueous solution comprising stevioside and the alpha-glucosyl sugar compound to form the desired alpha-glycosyl stevioside.

Preferably, the aqueous solution comprising stevioside and the alpha-glucosyl sugar compound is prepared by heating to dissolve the stevioside and is usually adjusted to give a stevioside concentration of from 0.1 to 20% (w/w) and an alpha-glucosyl sugar compound concentration of from 1 to 50% (w/w). The preferable ratio of alpha-glucosyl sugar compound to stevioside ranges from 0.5 to 500, d.s.b.

The pH and temperature of the reaction solution should be in the ranges that enable the formation of alpha-glycosyl stevioside by the action of alpha-glucosyltransferase(s). Usually a pH of 3—10 and a temperature of 20—80°C are used. In the reaction, the amount of enzyme used is closely related to the reaction duration. Therefore, from an economic point of view, usually an amount of enzyme sufficient to complete the reaction in about 5—8 hours is chosen.

The reaction solution in which alpha-glycosyl stevioside is thus formed can be used directly as the sweetener composition. If desired, the reaction solution is heated to inactivate the enzyme, filtered, deionised by passage of the filtrate through ion exchange resins (e.g. H type strongly acidic ion exchange resin and OH type weakly basic ion exchange resin), and concentrated into a syrupy sweetener composition, or the concentrate may be dried and pulverised to yield a powder sweetener composition. Any known method in the art for concentration, drying and pulverizing, e.g. vacuum

evaporation, vacuum drying and spray drying, may be used. The sweetness of the resulting alpha-glycosyl stevioside-containing sweetener composition is generally roughly equal to or slightly weaker than the sweetness imparted by a composition in which stevioside of the same solid weight is used for the reaction. Moreover, the sweetness of the new sweetener is mild, soft, round and pleasant, hardly imparting bitterness or astringency, and has no lingering unpleasant aftertaste.

The resultant alpha-glycosyl stevioside and unreacted stevioside present in the syrupy sweetener compositions of the invention do not crystallize on prolonged storage. The powder sweetener composition of the invention is a so-called powder solid solution wherein the resultant alpha-glycosyl stevioside, unreacted stevioside, reacted and unreacted-alpha-glucosyl sugar compounds are mutually dissolved. Accordingly, the water solubility of the powder sweetener composition is so high that it will dissolve instantly with no limitation; it dissolves freely even at high concentration to form a syrup or a paste.

As described above, the sweetener composition of the invention is different from conventional stevioside products or simple mixtures comprising stevioside and other sweeteners in that it dissolves freely without requiring heating due to its extremely high solubility. As will be described in detail below, this feature can be utilized with outstanding results when used to sweeten powdered instant foods, first foods, convenient foods and ingredients thereof.

The sweetener composition of the invention may be used alone as a seasoning to sweeten foods and drinks. If desired, the sweetener composition may be admixed with other sweeteners, e.g. starch syrups, glucose, maltose, isomerized sugar, sucrose, honey, maple sugar, sorbit, maltitol, dihydrochalon, L-aspartyl L-phenylalanine methyl ester, saccharin, alanine and glycyrrhizin, fillers, e.g., dextrins, starch and lactose, flavouring agents, and colouring agents.

The powdered sweetener compositions of the invention may be used directly in powder form or be molded into granular, spherical and tablet forms after adding suitable fillers and excipients, whereas the concentration of the syrupy sweetener compositions can be adjusted to meet the final use, and the syrup can be packed in containers or vessels of any shape.

Since, as described above, the sweetness of the sweetener composition of the invention is roughly equal to or slightly weaker than the sweetness imparted by a composition in which stevioside of the same solid weight is used for the reaction, the sweetness varies with the ratio of stevioside to alpha-glucosyl sugar compound used in the reaction. The sweetness attained with using a stevioside to alpha-glucosyl sugar compound ratio of about 1:50—100 is generally equal to that obtained from sucrose, d.s.b.

If this ratio exceeds about 100 and especially when gelatinized starch, partial starch hydrolyzate or maltodigosaccharide is used as alpha-glucosyl sugar compound, the resulting sweetness will be weaker than that of sucrose, d.s.b. Accordingly, such sweetener compositions can be used as a sweetener which imparts desirable texture such as thickness, viscosity, consistency, weight or body to foods, drinks, confectionery and pharmaceuticals and which reduces the sweetness of the final products.

On the other hand, if the said ratio is lower than about 50, the sweetness of the new sweetener will be, generally, higher than that of sucrose, d.s.b., and the sweetness intensifies inversely proportionally as the ratio decreases. Removal of the alpha-glucosyl sugar compound portion from the sweetener prepared in accordance with the invention will increase the sweetness of the sweetener to about 50—100 times of that of sucrose. Further the employment of such sweetness-intensified sweetener for sweetened foods, drinks and confectionery will lower their calorific values since a substantially lower amount of the present sweetener is required to obtain the sweetness attained with sucrose. In other words, the sweetener compositions of the invention can be used as low-calorific dietary sweeteners for obese persons, diabetics and for those whose calorific intake needs to be restricted, as well as for sweetening low calorific dietary foods, drinks and confectionery, i.e., the so-called beauty diets, health diets and other diets.

Moreover, the sweetener composition of the invention can also be used as a low-cariogenic or low-dental caries inducing sweetener since it is not readily fermentable by cariogenic microorganisms. It can be used in low cariogenic foods and drinks including confectionery such as chewing gum, chocolate, biscuits, cookies, caramel, toffee and candy; in soft drinks such as colas, juices, coffee, tea and lactic-acid beverages; in alcoholic beverages; as well as in dentifrices, gargles and pharmaceuticals.

Since the sweetener of the invention is compatible with and harmonizes well and desirably with food ingredients or additives which have other tastes such as sourness, saltiness, astringency bitterness and palatability to improve the taste and quality of the final products, and is highly acid and thermal resistant, it may be used freely in foods, drinks and confectionery, besides the above described products, in general, giving favourable results and improving their palatability.

Furthermore, the sweetener may be used as a palatability improver for feeds intended for domestic animals, cattle, horses, poultry, fish, honey bees and silk worms.

The sweetener may be also used for sweetening various products of tobacco, confectionery, cosmetics and pharmaceuticals of various forms (in solid, paste, liquid) such as lipstick, dentifrice, mouth refresher, gargles, bad-breath remover tablet, troche, sugar coating drops of cod-liver oil, medicine of oral administration, or freely used as gustatory improving agent, taste improver.

In order to incorporate the sweetener composition of the invention in the above described foods, drinks, confectionery, feed, cosmetics, dentifrices, and pharmaceuticals, any known method in the art may be chosen, for example, mixing, kneading, dissolving, dipping or immersing, permeance, dispersing, coating, spraying or infusing processes.

5 The following Experiments and Examples illustrate the present invention.

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Experiment 1. Preparation of Sweetener Composition 1—(1) Preparation of alpha-glucosyltransferase

10 A strain of *Bacillus stearothermophilus* FERM—P No. 2222 was inoculated into 10 liters of a sterilized culture medium containing 2% (w/v) soluble starch, 1% (w/v) ammonium nitrate, 0.1% (w/v) dipotassium hydrogen phosphate, 0.05% (w/v) magnesium sulfate, 7H₂O, 0.5% (w/v) corn steep liquor, and 1% (w/v) calcium carbonate, and the mixture was incubated at a temperature of 50°C for three days with aeration and stirring. The resultant culture broth was centrifuged and the supernatant was salted-out with 0.7 saturation ammonium sulfate, whereby a crude enzyme preparation with an activity of about 80,000 units was obtained. The enzymatic activity is assayed as follows. A mixture prepared by adding 0.2 ml of an enzyme solution to 5 ml of a solution containing 0.3 g/dl soluble starch, 0.02 M acetate buffer, pH 5.5 and 10⁻³M CaCl₂ was allowed to react at 40°C for 10 minutes. A 0.5 ml aliquot of the reaction mixture was collected poured into 15 ml of 0.02 N H₂SO₄ to terminate the reaction and assayed on optical density at 660 nm after addition of 0.2 ml of 0.1 N I₂—KI solution. One unit of activity was designated as the amount of enzyme that caused disappearance of iodine stain in 15 mg soluble starch at 40°C within 10 minutes.

20 1—(2) Reaction.

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25 "Commercialized stevioside", Stevia Sugar (Morita Kagaku Kogyo Co., Ltd., Osaka, Japan), 200 g, consisting of roughly equal amounts of purified stevioside and lactose, hereinafter referred to as "commercialized stevioside" and maltodextrin, D.E. 30, 1000 g, were dissolved with heating in 3,000 ml of water. After cooling to 60°C to the solution was added 1,000 units of crude cyclodextrin glucanotransferase obtained in Experiment 1—(1), adjusted to pH 6.0 and allowed to incubate at a temperature of 60°C for 40 hours. After holding the reaction solution at a temperature of 95°C for 10 minutes to inactivate the enzyme by heating (the resultant preparation corresponds to Sample No. 3 in Table 1), the filtrate obtained by filtration was decolorized with a small amount of activated carbon and then delonised by passage through the ion exchange resin Amberlite (Registered Trade Mark) IR—200C (H Type) and Amberlite IRA—93(OH type) (Rohm & Hass Co., Philadelphia, U.S.A.) at SV 2. Subsequently, the solution was concentrated at a temperature of 70°C or below under reduced pressure and dried into powder form (the resultant preparation corresponds to Sample No. 4 in Table 1). The controls were obtained by similar processes using dissolution by heating such as the reaction process and inactivation process by heating; the starting material used in the controls is given in Table

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TABLE 1

Sample	No. 1 (control)	No. 2 (control)	No. 3 (product of the invention)	No. 4 (product of the invention)
Starting material	200 g "commercialized stevioside"	200 g "commercialized stevioside" + 1,000 g maltodextrin + 1,000 units enzyme prein- activated by heating	200 g "commercialized stevioside" + 1,000 g maltodextrin + 1,000 units enzyme	200 g "commercialized stevioside" + 1,000 g maltodextrin + 1,000 units enzyme
Remarks	prepared similarly as Sample No. 3	prepared similarly as Sample No. 3	crude liquid sweetener obtained by heating	powder sweetener prepared by purifying and drying Sample No. 3

Experiment 2. Taste Tests**2—(1) Comparison of Sweetness**

The concentration of every taste test solution was adjusted with reference to the preliminary test results to approximate the sweetness. More particularly, the concentration of the controls was adjusted to give a "commercialized stevioside" concentration of 0.13% (w/v), while the concentration for the sweetener of the invention was adjusted to correspond to a concentration of 0.20% (w/v) of "commercialized stevioside".

The test panel members gave their sweetness sensory results with reference to a sucrose (granulated sugar) solution of a certain concentration, stating whether they found the test solution sweeter or less sweet than the sucrose solution. The tests were performed at room temperature with a panel of 20 members. The results are listed in Table 2.

TABLE 2

Sample Comparison of sweetness Sucrose concentration % (w/w)	No. 1 (control)			No. 2 (control)			No. 3 (product of the invention)			No. 4 (product of the invention)		
	sweeter	same	less sweet	sweeter	same	less sweet	sweeter	same	less sweet	sweeter	same	less sweet
6	13	5	2	15	3	2	/	/	/	/	/	/
7	10	2	8	8	5	7	/	/	/	20	0	0
8	4	4	12	3	3	14	16	2	2	15	4	1
9	1	3	16	1	2	17	10	5	5	8	6	6
10	0	2	18	0	1	19	7	4	9	0	6	14
11	/	/	/	/	/	/	2	3	15	0	6	14
12	/	/	/	/	/	/	0	2	18	0	2	18

Note: Figures indicate number of answers.

As the results shown in Table 2 indicate, the respective ratios of those who answered Sample No. 1 "sweeter" than the 6% and 7% sucrose solutions to those who answered "less sweet" were 13:2 and 10:8, whereas the ratios were 4:12 and 1:16 for the 8% and 9% sucrose solution. Based on the close ratio it was concluded that Sample No. 1 and the 7% sucrose solution were approximately equal in

5 sweetness.

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Similarly, Sample No. 2 and the 7% sucrose solution were rated approximately equally sweet based on the "sweeter" to "less sweet" ratio. Accordingly the sweetening power of Sample No. 1 was concluded as corresponding to the 7% sucrose solution. Similarly, the sweetening power of Sample No. 3 was determined as corresponding to that of the 10% sucrose solution, and Sample No. 4 to the 9%

10 sucrose solution.

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Accordingly, no differences were noted between the sweetening powers of Samples No. 1 and No. 2 based on the amount of "commercialized stevioside" used in both Samples, d.s.b., and the sweetening power was calculated as about 53 times of that of sucrose. Samples No. 3 and No 4 were also calculated as having sweetening powers of 50 and 45 times respectively of that of sucrose, while

15 they were roughly equal to or slightly lower than those of Samples No. 1 and No. 2. In other words, the sweetening powers of the sweetener prepared in accordance with the invention are approximately equal to or slightly lower than that of stevioside in the amount used as material.

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2—(2) Comparison Tests of Sweetness Quality

Comparison tests were performed with controls (Samples No. 1 and No. 2) and sweeteners of the invention (Samples No. 3 and No. 4) for their sweetness quality.

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Aqueous solutions of the above Samples were prepared to give a sweetness corresponding respectively to those of the 5%, 10% and 15% aqueous sucrose solutions by calculation on the basis of the result of the Experiment 2—(1). The test panel of 20 members were instructed to choose at room temperature among the test aqueous solutions of Samples No. 1—4 the respective samples with the

25 most desirable and the most undesirable sweetness quality. The results are listed in Table 3.

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TABLE 3

Sample Sweetness quality Sweetness	No. 1 (control)		No. 2 (control)		No. 3 (product of the invention)		No. 4 (product of the invention)	
	most desirable	most undesirable	most desirable	most undesirable	most desirable	most undesirable	most desirable	most undesirable
5% sucrose solution	0	10	0	10	4	0	16	0
10% sucrose solution	0	8	0	12	6	0	14	0
15% sucrose solution	0	11	0	9	7	0	13	0

Note: Figures indicate number of answers

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As apparent from the results in Table 3, the sweetness qualities of the controls, Samples No. 1 and No. 2, were noted as most inferior at every degree of sweetness, thus no differences were noted between Samples No. 1 and No. 2, whereas it is clear that the sweetness qualities of Samples No. 3 and No. 4 were superior at every degree of sweetness. Especially the further purified sweetener, Sample No. 5 4 was observed as being slightly superior to Sample No. 3 in sweetness quality. The responses from the panel members on sweetness quality are listed in Table 4.

TABLE 4

Sample Corresponding sweetness	No. 1 (control)	No. 2 (control)	No. 3 (product of the invention)	No. 4 (product of the invention)
5% sucrose solution	Sweet, bitter, astringent, no lingering after taste, difficult to use intact	Sweet, bitter, astringent, no lingering after taste, difficult to use intact	Sweet, light, soft, round, pleasant, similar to sucrose, no lingering after taste advantageously intact may be used	Sweet, soft, round, pleasant, similar to sucrose, no lingering after taste. advantageously intact may be used
10% sucrose solution	Sweet, sharp, stimulant, strong, bitter, astringent, no lingering after taste, difficult to use intact	Sweet, sharp, stimulant, strong bitter, astringent, no lingering after taste, difficult to use intact	Sweet, soft, round, pleasant, similar to sucrose, no lingering after taste, advantageously intact may be used	Sweet, soft, round, pleasant, similar to sucrose, no lingering after taste, advantageously intact may be used
15% sucrose solution	Sweet, sharp, stimulant, bitter, astringent, no lingering after taste, difficult to use intact	Sweet, sharp, stimulant, bitter, astringent, no lingering after taste, difficult to use intact	Sweet, soft, round, pleasant, similar to sucrose, no lingering after taste, advantageously intact may be used	Sweet, soft, round, pleasant, similar to sucrose, no lingering after taste, advantageously intact may be used

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As the above test results show, unlike the conventional stevioside or mixture of stevioside and other sweeteners, the sweeteners of the invention have a mild, soft, round and pleasant sweetness closely resembling that of sucrose without an undesirable lingering after-taste, and are superlative sweeteners which impart to the mouth directly a pleasant and enjoyable sweetness.

5 Experiment 3. Comparison of Solubility

Portions of Samples No. 2 and No. 3 prepared in Experiment 1—(2) were transferred into test tubes, allowed to stand in a room maintained at 4°C for 15 days and their water solubility was compared. As illustrated in the drawings of Figure 1, Sample No. 2 crystallized and became white turbid, whereas Sample No. 3 of the invention remained transparent and had a remarkably improved

10 water solubility.

Samples No. 4 which was obtained by purifying, concentrating and drying Sample No. 3 was a so-called "solid solution" wherein constituents such as alphasglycosyl stevioside, stevioside, alpha-glucosyl sugar compounds were mutually dissolved and their solubility was so high that it instantly dissolved in water and that even at high concentration it freely dissolved to form into a paste.

15 Experiment 4. Identification of Alpha-glycosyl Stevioside

Five grams of Sample No. 4 obtained in Experiment 1—(2) were added to a solvent system of water-saturated n-butanol (200 ml) and water (200 ml) placed in a separating funnel, and alpha-glucosyl sugar compounds and glycosides present in Sample No. 4 were extracted, portioned with the solvent system, and the n-butanol layer was collected. After drying the obtained solid under reduced pressure to remove n-butanol, the dried product was dissolved in a small amount of methanol, then excess ethyl ether was added, and the resultant precipitate was collected, dried under reduced pressure, and pulverized, whereby 500 mg of powder product (Sample No. 5) was obtained. Sample No. 5 dissolved very easily in water, and was an odorless, white-colored and neutral substance that had a mild, soft round and pleasant sweetness. It also dissolved partially in lower alcohols such as methanol, ethanol and n-butanol, but was not readily soluble in chloroform and ethyl ether. The infrared spectrum of Sample No. 5 (KBr tablet method) is shown in Figure 2. Also its ultraviolet spectrum was recorded, but no characteristic absorbance spectrum was noted.

An aqueous solution prepared by dissolving a portion of Sample No. 5 in a small amount of water; an aqueous solution of Sample No. 1 purified similarly as in the case of Sample No. 5 and a solution of D-glucose as a control were spotted on a thin layer plate of Silica gel 60 (Merck & Co., Inc., New Jersey, U.S.A.), and one dimensional development was carried out in a solvent system of chloroform methanol and water (30:20:4). Then the thin layer plate was air-dried sufficiently. A 0.1 M acetate buffer solution, pH 4.8, in which commercially available crystalline glucoamylase was dissolved, was sprayed on the thin layer plate and then the plate was allowed to stand at 50°C for two hours taking precaution to prevent drying. Thereafter the plate was air-dried sufficiently and two dimensional development of the plate was carried out using the same solvent system. After drying, the plate was sprayed with a methanol solution containing 50% sulfuric acid, heated and developed to give the chromatogram shown in Figure 3. As can be seen from Figure 3, in addition to a stevioside spot (Rs 1.00), small spots of rebaudioside A (Rs 0.88) and rebaudioside B (Rs 1.17) were confirmed by the result of one dimensional development of Sample No. 1 as a control. Whereas the result of the chromatograph Sample No. 5 shows that, besides spots of stevioside and rebaudiosides, six new spots of substances, in the decreasing order of Rs value, i.e., Rs 1.09, Rs 0.80, Rs 0.67, Rs 0.62, Rs 0.51 and Rs 0.48, were developed, which were designated as (a), (b), (c), (d), (e) and (f) respectively.

The result of two dimensional development obtained wherein glucoamylase was allowed to act on Sample No. 5 revealed that spot (Rs 1.17) of rebaudioside B and spot (Rs 0.45) of D-glucose originated from substance (a); spot (Rs 1.00) of stevioside and spot (Rs 0.45) of D-glucose, from substance (b); spot of substance (b), in addition to spot (Rs 1.00) of stevioside and spot (Rs 0.45) of D-glucose, from substance (d); spot of substance (b), in addition to spot (Rs 1.00) of stevioside and spot (Rs 0.45) of D-glucose, from substance (f). Similarly, spot (Rs 0.88) of rebaudioside A and spot (Rs 0.45) of D-glucose originated from substance (c), spot of substance (c), in addition to spot (Rs 0.88) of rebaudioside A and spot (Rs 0.45) of D-glucose, from, substance (e).

It is apparent from the results that substances (a)—(f) were decomposed respectively by the action of glucoamylase into D-glucose derivatives of stevioside or rebaudioside, and finally into D-glucose and stevioside, or D-glucose and rebaudioside. Therefore it was concluded that substance (a) was a substance in which D-glucose was alpha-linked with rebaudioside B, and that substances (b), (d) and (f) were substances in which one mole of stevioside was also alpha-linked with one or more moles of D-glucose. In other words, it was concluded that the spots were alpha-monoglucosyl stevioside, alpha-diglucosyl stevioside and alpha-triglucosyl stevioside, respectively. Similarly, it was concluded that substances (c) and (e) were alphamonoglucosyl rebaudioside A and alpha-diglucosyl rebaudioside A, respectively.

Substances (a)—(f) decomposed into stevioside and D-glucose, or rebaudioside and D-glucose when a partially purified alpha-glucosidase extracted from pig liver was allowed to react similarly on them. Substance (f) was found to be easily decomposable into substance (b) and maltose by the action of commercially available crystalline beta-amylase.

From these results, it was concluded that substances newly formed by the action of alpha-

glucosyl-transferase were substances in which one mole of stevioside was alpha-linked with one or more moles of D-glucose, or in which one mole of rebaudioside was alpha-linked with one or more moles of D-glucose. This suggested that these substances may be decomposable into stevioside or rebaudioside *in vivo* in human or animals.

5 A sample prepared similarly as Sample No. 5 was applied to silica gel column chromatography using a solvent system of chloroform, methanol and water (60:30:5) to isolate substance (b), which was then dried to a powder. The substance was an extremely readily water soluble, odorless and white colored sweetener with a mild, soft, but intense sweetness and a pH value around neutrality. It was partially soluble in lower alcohols such as methanol, ethanol and n-butanol, but not readily soluble in chloroform and ethyl ether. The infra-red spectrum of substance (b) by the KBr table method is shown in Figure 4. 10

Since Sample No 5 possesses an excellent sweetness, free of a lingering aftertaste and is extremely readily water soluble, similarly as Samples No. 3 and No. 4 employed in the Experiment 2—(2), the purposes of the invention which is to eliminate the disadvantages of stevioside, can be achieved by allowing alpha-glucosyltransferase to react on an aqueous solution comprising stevioside and alpha-glucosyl sugar compound to form an alpha-glycosyl stevioside-containing composition. 15

Example 1

Five liters of a sterilized culture medium containing 4% (w/v) maltose, 0.1% (w/v) potassium dihydrogen phosphate, 0.1% (w/v) ammonium nitrate, 0.05% (w/v) magnesium sulfate, 7H₂O, 0.05% (w/v) potassium chloride, 0.2% (w/v) polypeptone and 1% (w/v) calcium carbonate which was separately dry heat sterilized and added aseptically to the medium on inoculation was inoculated with a strain of *Mucor javanicus* IFO 4570 and incubated at a temperature of 30°C for 44 hours with aeration and stirring. Thereafter 480 g of the wet mycelia obtained from the culture broth was added to an M/2 acetate buffer solution pH 5.3 in 4 M urea 5 liters, the resulting mixture was allowed to stand at a temperature of 30°C for 40 hours. the supernatant of the mixture was dialyzed overnight against running tap water, saturated to 0.9 ammonium sulfate, allowed to stand at a temperature of 4°C overnight, and then the precipitates were collected by centrifugation. After suspending the precipitate in 100 ml of acetate buffer (pH 6.0), the mixture was centrifuged and the supernatant was used as alpha-glucosidase (E.C.3.2.1.20). 20

30 "Commercialized stevioside" (15 g) and malto-dextrin (D.E. 40, 300 g) were dissolved in one liter of heated water, and the mixture was cooled to 50°C, added the above alpha-glucosidase solution and allowed to stand at pH 6.0 and 50°C for 24 hours. The reaction solution was heated to inactivate the enzyme therein and then filtered. The filtrate was deionised by passing it through the ion exchange resin Amberlite IR—120B (H Type) and Amberlite IRA—94 (OH Type) (Rohm & Haas Co., Philadelphia U.S.A.), and was concentrated under reduced pressure. The resulting solution gave a liquid sweetener with a water content of 30% (w/w). The yield of the liquid sweetener was 97%. d.s.b., against material. The sweetener was about two times as sweet as sucrose, d.s.b., free of a lingering unpleasant aftertaste. Therefore, it can be freely used in sweetening various foods, drinks and confectionery. 35

Particularly it can be used as a low cariogenic sweetener because oral cariogenic microorganisms do not convert it into a water insoluble glucan. 40

Example 2.

A strain of *Bacillus megaterium* FERM—P No. 935 was inoculated into 5 liters of the culture medium of Experiment 1—(1), and the mixture was incubated at 28°C for three days with aeration and stirring. After the incubation was finished, the resulting culture broth was centrifuged, and the supernatant was salted-out with 0.7 saturation ammonium sulfate and then centrifuged, whereby a precipitate was obtained. 45

The precipitate contained 300,000 units of cyclodextrin glucanotransferase (E.C.2.4.1.19) as assayed by the method of measurement described in Experiment 1—(1). To a 30% (w/w) sweet potato starch slurry (pH 6.0) was added a commercially available liquefying enzyme, 0.2% per dry solid of starch, and the mixture was liquefied continuously at 85—95°C. The decomposition of the mixture was effected at 80°C to a D.E.20, and the liquefying enzyme was inactivated by heating. "Commercialized stevioside" was dissolved in the liquefied starch, to give a mixture of one part of "commercialized stevioside" and three parts of partial starch hydrolyzate, d.s.b. The mixture was cooled to 50°C, cyclodextrin glucanotransferase was added (10 units per gram starch) and incubated at 50°C and pH 6.0 for 48 hours. After inactivating the enzyme in the reaction mixture by heating, the resultant mixture was purified similarly as in Example 1, concentrated under reduced pressure, dried and pulverized, whereby a powder sweetener was obtained in a yield of about 95%, d.s.b. Since the sweetener was only slightly hygroscopic, it was easy to handle. But its water solubility was so high, and it dissolved easily in cold water, and even at high concentration to form a paste. 50

60 The sweetener was about 15 times sweet as sucrose, d.s.b., and it possessed a mild, soft, round and pleasant sweetness, free of an unpleasant lingering aftertaste. This sweetener can be used whenever sweetening is necessary, but especially it is suitable as a dental caries preventive and low calorie sweetener. 60

Example 3.

65 Potato starch (300 g) and "commercialized stevioside" (100 g) were added to one liter of water, 65

and to the mixture was added commercially available bacterial saccharifying alpha-amylase (E.C.3.2.1.1.) (Seikagaku Kogyo Co., Ltd., Tokyo, Japan), in the amount of 10 units (as assayed by the method of Experiment 1—(1)) per gram of starch and heated with stirring to 80°C. After the liquefaction of the starch ended, the mixture was cooled to 60°C and allowed to stand for two days.

- 5 After inactivating the alpha-amylase present in the resulting mixture by heating, the mixture was purified in the same manner as in Example 1, concentrated under reduced pressure, dried and pulverized, whereby a powder sweetener was obtained in a yield of about 95%, d.s.b. 5

The sweetener has similar qualities as those of the sweetener obtained in Example 2, and also it can be directed to various uses.

10 **Example 4.** 10

A liquefied starch solution (30% (w/w) and of D.E. 15) was prepared by the same method as in Example 2. Into the liquefied starch, was added and dissolved "commercialized stevioside" and sucrose of a third amount of material starch, respectively, and then added 2 units of cyclodextrin glucanotransferase (E.C.2.4.1.19) per gram starch, d.s.b., and the mixture was subjected to reaction at 60°C for 24 hours. After inactivating the enzyme by heating, the resulting mixture was purified by the method in Experiment 1—(2), concentrated and dried, whereby a powder sweetener was obtained. 15

The resultant sweetener was a mixture containing alpha-glycosyl stevioside, alpha-glycosyl sucrose, etc., wherein the tastes of these compounds were well harmonized and exhibited an excellent sweetness quality. The sweetener was about 15 times as sweet as sucrose. It may be used as a sweetener for many uses. Especially, since the reducing power of the sweetener is low, it is a suitable sweetener for products containing amino acids and proteins such as foods, drinks, confectionery and pickles which undergo heating during production. Since the sweetener is hardly converted into water-insoluble glucan by oral cariogenic microorganisms, it is a suitable low cariogenic sweetener for foods and drinks. 20

25 **Example 5.** 25

The same reaction as described in Experiment 1—(2) was carried out except using a greenish ochre colored crude stevioside, STV—B, (Ikeda Tohka Industries Co., Ltd., Fukuyama, Japan) containing only about 50% stevioside instead of "commercialized stevioside".

- 30 It was found that the sweetener had a remarkably more improved sweetness quality than that of the sweetener obtained by using purified stevioside. The sweetener was analyzed. A large amount of alphasglycosyl rebaudioside A and of alpha-glycosyl rebaudioside B were detected besides alpha-glycosyl stevioside. 30

The sweetener is about 10 times as sweet as sucrose and can be used in sweetening various foods, drinks confectionery and pharmaceuticals.

- 35 In the production of the sweetener, elimination of colors of the starting material presents some difficulties but may be used advantageously in products such as soy sauce, pickles or salted vegetables, and preserved foods boiled down in soy sauce wherein coloring is not so important because it uses as starting material crude stevioside which can be produced at a cheaper cost and in a larger amount than purified stevioside. 35

40 **Example 6** 40

One percent seed culture of *Leuconostoc mecenteriodes* IAM 1151 was inoculated on 10 liters of a culture medium containing 4% (w/v) sucrose, 0.5% (w/v) yeast extract, 0.8% (w/v) potassium hydrogen phosphate, 2.4% (w/v) dipotassium hydrogen phosphate, 0.02% (w/v) magnesium sulfate 7H₂O and 0.5% (w/v) purified stevioside, and the mixture was subjected to stationary culture at 25°C for 24 hours. The resulting culture broth was centrifuged and the supernatant was purified similarly as the reaction solution in Example 1, and concentrated, whereby a liquid sweetener, water content 30%, was obtained in a yield of about 60%. 45

The sweetener possessed a mild, soft, round and pleasant sweetness which was about 20 times as sweet as that of sucrose and did not effect crystallization of stevioside. Since the sweetener is highly viscous, it imparts a sweetness and a desirable viscosity to products wherein it is used. It is advantageous for sweetening, e.g., juice, syrup, liquor and pickles or salted vegetables. 50

Further, the above seed culture was inoculated on the same culture medium as above, but from which purified stevioside was removed, and the mixture was incubated similarly. The thus obtained culture broth was centrifuged, and to the supernatant was added calcium phosphate gel. Then the mixture was dialyzed against tap water, and the dialyzed solution was centrifuged again to recover the calcium phosphate gel. The dextran sucrose (E.C.2.4.1.5) solution (100 ml) obtained from eluting and concentrating a suspension of the gel in a 0.2 M sodium dihydrogen phosphate solution saturated to 0.35 ammonium sulfate was allowed to react on 5 liters of a solution containing 4% (w/v) sucrose and 0.5% (w/v) purified stevioside at pH 5.3 and 30°C for 10 hours. After inactivation by heating, the reaction solution was purified, and concentrated in the same manner described above, whereby a liquid sweetener was obtained in a yield of about 90%, d.s.b. Since the alpha-glycosyl stevioside content in the sweetener was decomposed by isomaltodextrase (E.C.3.2.1.94) into isomaltose, stevioside and alpha-monoglucosyl stevioside, it was concluded that the sweetener was a mixture in which one mole of stevioside was alpha-linked with one or more moles of D-glucose. 55 60

Example 7

A liquid sweetener prepared by dissolving the powder sweetener (26 g) obtained in Example 2 in 1 k.g. of Malbit (food grade maltitol syrup, water content 25%, Hayashibara Co., Ltd., Okayama, Japan) had an excellent sweetness quality, and the sweetness was equal to that of sucrose and the calorific value of the sweetener was about one twentieth of that of sucrose.

The sweetener is suitable for preparing low calorie foods, drinks and confectioneries for those whose calorific intakes are restricted, e.g., obese persons, diabetics, the figure-conscious and weight watchers, and it can be also used directly as a table syrup. Since neither acid nor water insoluble glucan is produced by oral cariogenic microorganisms, it is suitable for preparing low cariogenic foods and drinks.

Example 8

Glucose (960 g), sucrose (20 g) and the powder sweetener (20 g) prepared in Example 3 were mixed uniformly, and the mixture was pulverised into a powder sweetener.

Its sweetness was roughly equal to that of sucrose and its sweetener quality was excellent.

Further, the sweetener was soluble in cold water.

A chilled aqueous solution of the sweetener itself may be used as a delicious soft drink. It was concluded that the excellent sweetness quality of the powder sweetener mixture is due to the synergic effect of the above three constituents, i.e., glucose, sucrose and powder sweetener.

Example 9

A complex sweetener was prepared by dissolving 100 g of powder sweetener Sample No. 4 obtained in Experiment 1—(2) in 20 ml of water and by admixing uniformly 1 kg of honey thereto.

The sweetener was about two times as sweet as sucrose and its sweetness quality was very excellent. Further the presence of Sample No. 4 intensified the fragrance of honey. It can be freely used as a sweetener for beauty drinks and health diets as well as a taste improver for herb medicines.

25 CLAIMS

1. Alpha-glycosyl stevioside, or a composition comprising an alpha-glycosyl stevioside, each for use as a sweetener.

2. A sweetener according to Claim 1, wherein the alpha-glycosyl stevioside is alpha-monoglucosyl stevioside, alpha-diglucosyl stevioside and/or alpha-triglucosyl stevioside.

3. A sweetener according to Claim 1 or Claim 2, which composition is obtained by subjecting an aqueous solution comprising stevioside and at least one alpha-glycosyl sugar compound to the action of an alpha-glucosyltransferase.

4. A sweetener according to Claim 3, wherein the alpha-glucosyltransferase is one or more of an alpha-glucosidase, an alpha-amylase, a cyclodextrin glucanotransferase, a dextranase, a dextrin-dextranase and an amylosucrase.

5. A sweetener according to Claim 3 or 4, wherein the alpha-glucosyl sugar compound is one or more of maltooligosaccharide, a starch, a partial starch hydrolyzate, a cyclodextrin and sucrose.

6. A sweetener according to Claim 1 substantially as described in any one of the foregoing Experiments 1 to 4 and Examples 1 to 9.

7. A process for producing a sweetener composition comprising an alpha-glycosyl stevioside which process comprises subjecting an aqueous solution comprising stevioside and at least one alpha-glucosyl sugar compound to the action of an alpha-glucosyltransferase to produce, as the sweetener composition, an alpha-glycosyl stevioside containing composition.

8. A process according to Claim 7, wherein the alpha-glucosyltransferase is one or more of an alpha-glucosidase, an alpha-amylase, a cyclodextrin glucano-transferase, a dextranase, a dextrin-dextranase and an amylosucrase.

9. A process according to Claim 7 or Claim 8, wherein the alpha-glucosyl sugar compound is one or more of a maltooligosaccharide, a starch, a partial starch hydrolyzate, a cyclodextrin and sucrose.

10. A process according to Claim 7, substantially as described in any one of the foregoing Experiment 1 and Examples 1 to 9.

11. A sweetener whenever obtained by a process as claimed in any one of Claims 7 to 10.

12. Food, drink, confectionary, tobacco, feedstuffs, cosmetics, dentrifices, and pharmaceuticals whenever sweetened by incorporation of a sweetener as claimed in any one of Claims 1 to 6 and 11.

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10. A process according to Claim 7, substantially as described in any one of the foregoing

Experiment 1 and Examples 1 to 9.

11. A sweetener whenever obtained by a process as claimed in any one of Claims 7 to 10.

12. Food, drink, confectionary, tobacco, feedstuffs, cosmetics, dentrifices, and pharmaceuticals whenever sweetened by incorporation of a sweetener as claimed in any one of Claims 1 to 6 and 11.

