CYCLOTETRAGLUCOSE AND CYCLOTETRAGLUCOSE SYRUP
Chemical and Technical Assessment

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1. Summary

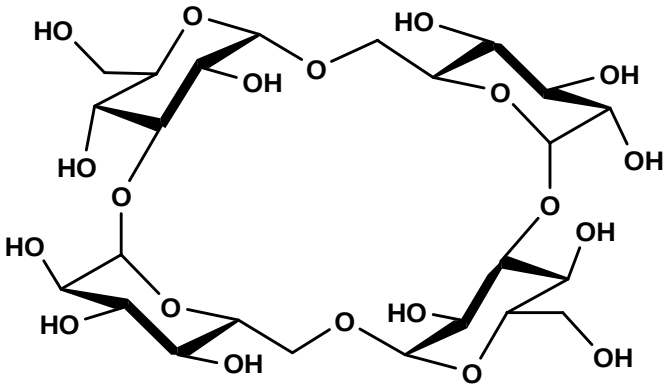
Cyclotetraglucose is a non-reducing cyclic tetrasaccharide comprised of four D-glucopyranosyl residues linked by alternating α(1→3) and α(1→6) glucosidic bonds. Cyclotetraglucose syrup is a mixture consisting of mainly cyclotetraglucose with branched cyclotetraglucose, mono- and disaccharides, and linear branched or not branched oligosaccharides. They are produced from hydrolyzed food-grade starch by the actions of a mixture of 6-α-glucosyltransferase and α-isomaltosyltransferase derived from *Sporosarcina globispora*, and cyclodextrin glucosyltransferase derived from *Bacillus stearothermophilus* from liquefied food-grade starch as the starting material by the joint actions of 6-α-glucosyltransferase and α-isomaltosyltransferase, both isolated from *Sporosarcina globispora.* Depending on the following steps, crystalline cyclotetraglucose and cyclotetraglucose syrup are produced separately. They are used as a carrier.

Cyclotetraglucose had not been evaluated by the Committee and it was placed on the agenda at the request of 37th session of CCFAC (2005) under the name cyclotetraose. The Committee considered that the name cyclotetraose was misleading as it suggests that the substance is a four-carbon sugar whereas it is actually a cyclic tetramer of glucose. The Committee therefore assigned it the name cyclotetraglucose. In reaching its decision the Committee took into account the principles on nomenclature elaborated at its 33rd meeting.

2. Description

Cyclotetraglucose is a non-reducing cyclic tetrasaccharide comprised of four D-glucopyranosyl residues linked by alternating α(1→3) and α(1→6) glucosidic bonds. The chemical name is *cyclo*[→6)-α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→3)- α- D-glucopyranosyl-(1→]. C.A.S. numbers have been allocated for the cyclotetraglucose pentahydrate (532945-76-9), monohydrate (532945-75-8) and anhydride (159640-28-5). Structure formula is shown in Figure 1.

Figure 1. Cyclotetraglucose



The synonyms are cyclotetraose, cyclic nigerosyl-1,6-nigerose, cycloalternan, cycloalternanotetraose. CT-11 is the Applicant's internal code name for cyclotetraglucose it is included here because may appear in some of the unpublished internal research reports.

Cyclotetraglucose syrup is a mixture consisting of mainly cyclotetraglucose with branched cyclotetraglucose, mono- and disaccharides, and linear branched or not branched oligosaccharides. The product contains 30 – 40% cyclotetraglucose on a dry matter basis.

Crystalline cyclotetraglucose and its aqueous solutions are odourless and essentially tasteless. A very slight sweetness may be perceived, but it is too slight to be of practical relevance, while, cyclotetraglucose syrup is slightly sweet because of coexistent saccarides.

Cyclotetraglucose occurs naturally in sake lees (i.e., the sediment that forms during rice wine production), in sake itself, and in the cells of *Saccharomyces cerevisiae* (Watanabe et al., 2004). Cyclotetraglucose has been synthesized for the first time by treating alternan, a linear oligosaccharide, with alternanase. In the large scale production it is produced by the joint action of 6-α-glucosyltransferase (6-GT) and α-isomaltosyltransferase (IMT), both isolated from *Sporosarcina globispora*, on liquefied starch.

3. Manufacturing

3.1. Manufacturing principle

Cyclotetraglucose is produced from liquefied food-grade starch as the starting material, by the actions of (i) an enzyme preparation comprised of a mixture of 6-GT and IMT derived from *Sporosarcina globispora (previously named as Bacillus globisporus*) (Yoon et al., 2001; Euzéby & Tindall, 2004) and (ii) cyclodextrin glucosyltransferase (CGTase; EC 2.4.1.19) derived from a strain of *Bacillus stearothermophilus*. The reaction is carried out at neutral pH and moderate temperature. Either crystalline cyclotetraglucose or cyclotetraglucose syrup is produced depending on the down-stream production steps.

3.2. Detailed Manufacturing

3.2.1 Production of pure crystalline cyclotetraglucose

In a first step, food-grade starch (e.g., tapioca starch, cornstarch) is liquefied using a food-grade thermostable α–amylase. For the second step, the pH of the liquefied starch solution (concentration approx. 20% w/w, 3.0% hydrolysis) is adjusted to about pH 6. Then, 6-GT and IMT containing enzyme preparation from *S. globisporus* as well as cyclomaltodextrin glucanotransferase are added. The mixture is reacted at about 45° for 72 hours whereupon the enzymes are inactivated by heat treatment.

In a next step, unreacted remaining maltosyl oligosaccharides and branched cyclotetraglucose derivatives are destroyed by incubation with food-grade α-glucosidase, glucoamylase and α-amylase at about pH 5.5 and 50° for 40 hours. After deactivation of these enzymes by heat treatment, the mixture is decolorized with activated carbon, filtered, concentrated under reduced pressure and desalted.

In order to separate cyclotetraglucose from sugars and saccharide-type by-products, the filtrate is subjected to chromatographic separation using suitable cation-exchange resins in the sodium form. The cyclotetraglucose containing fraction, which has a purity of about 90%, is collected and concentrated at elevated temperature under reduced pressure. Crystalline product precipitates upon cooling. The obtained cyclotetraglucose pentahydrate crystals are recovered by centrifugation and dried.

3.2.2. Production of cyclotetraglucose syrup

The first two steps of this production process are essentially the same as described for pure crystalline cyclotetraglucose in Section 3.2.1. However, the reaction mixture that results from the 6-GT and IMT treatment is, without prior deactivation of these two enzymes, treated with food-grade thermostable α-amylase which degrades unreacted maltooligosaccharides but not cyclotetraglucose or its branched derivatives to products of lower DP (Aga et al., 2002). The reaction mixture is then deactivated by heat treatment, decolorized with activated carbon, filtered, concentrated, desalted and subjected to a second decolouration before final filtration. The resulting syrup may be sold as such or it may be spray-dried to give cyclotetraglucose syrup solids.

3.3 Source organism of 6-GT and IMT

The conversion of liquefied starch to cyclotetraglucose was catalyzed by the joint action of two enzymes, namely 6-α-glucosyltransferase (6-GT) and α-isomaltosyltransferase. Both enzymes are secreted by *B. globisporus* in the culture fluid (Nishimoto et al., 2002; Aga et al., 2003).

B. globisporus was first described as a species by Larkin & Stokes (1967) who investigated psychrophylic strains of *Bacillus*. On the basis of a recent taxonomic re-evaluation, it has been proposed to transfer *B. globisporus* to the genus Sporosarcina as *Sporosarcina globispora* (Yoon et al., 2001).

A literature search in standard databases did not reveal any evidence for a toxigenic or pathogenic potential of *B.globisporus*, which is listed in risk group 1 of the German Ausschuss für Biologische Arbeitsstoffe (Committee for Biological Agents)(ABAS, 2006).

3.4 Production of 6-GT and IMT

For the preparation of 6-GT and IMT, *B. globisporus* strain N75 is grown in a culture medium containing food-grade dextrin, yeast extract, potassium and sodium phosphate, magnesium sulfate and calcium carbonate. Best growth is achieved at about 27-30°. When the desired enzyme activity has been reached in the culture broth, the cells are removed by filtration through a microfilter membrane. The filtrate is then purified and concentrated by ultrafiltration. The ultrafiltered culture supernatant was reported to have specific 6-GT and IMT activities of 100 and 320 U/g (d.s.), respectively (Aga et al., 2003). About half of the dry substance represents protein that can be precipitated with saturated ammonium sulfate.

3.5 Glycosidic by-products of cyclotetraglucose

Considering the complex composition of the raw material from which cyclotetraglucose is produced (i.e., liquefied starch), and the rather broad substrate specificity of the enzymes which are applied for its synthesis, a number of carbohydrate by-products are formed during the production of cyclotetraglucose (Nishimoto et al., 2002; Aga et al., 2004).

By transglycosylation reactions of 6-GT and IMT, 4-*0*-α-D-glycopyranosyl-cyclotetraglucose and 3-*0*-α-isomaltosyl-cyclotetraglucose are formed as the main cyclotetraglucose-type by-products. These branched cyclotetraglucose derivatives are debranched by α-glucosidase (Aga et al., 2002). Another three quantitatively less important branched cyclotetraglucoses were identified (Aga et al., 2004). In addition, glucose, small amounts of fructose, linear disaccharides (isomaltose, maltose, trehalose, nigerose, kojibiose, neotrehalose), trisaccharides (isomaltotriose, panose, maltotriose) and a multitude of other unidentified

saccharides, which occur in very small amounts only, are present in the reaction mixture that results from the 6-GT/IMT step.

In the production of pure crystalline cyclotetraglucose, most of the higher oligosaccharides are degraded by the action of α-glucosidase, glucoamylase and α-amylase in the last enzymatic step. The resulting glycosidic break-down products are then removed almost quantitatively during the ensuing purification steps. However, with a purity of not less than 98%, trace amounts of these carbohydrate by-products may still be present in the final crystalline cyclotetraglucose product.

In the production of cyclotetraglucose syrup, only α-amylase is applied for depolymerising oligosaccharides to smaller fragments. The branched cyclotetraglucose species remain intact. Because the reaction mixture is not fractionated by ion-exchange chromatography, the whole spectrum of glycosidic products is present in the final product.

4. Characterization

4.1. Composition

Crystalline cyclotetraglucose is pentahydrate. Depending on dryness it changes to monohydrate or anhydrous. The cyclotetraglucose product has a purity of at least 98% as dry basis. Table 1 show the batch analyses of pure crystalline cyclotetraglucose, which were checked by the applicant for purpose of the internal quality control.

The liquid syrup has at least 70% of solid content and between 30-40% of cyclotetraglucose content as dry basis. Branched cyclotetraglucose species bring the total content of cyclotetraglucose-type substances to 45-55%. Mono-, di- and tri-saccharide represent about 15-20%. The remaining fraction of about 30% consists of a variety of enzymatic breakdown products of starch (i.e. saccharides) which have not been identified specifically. Quantitative data on the composition of one batch of cyclotetraglucose syrup are presented in Table 2. The batch analyses of cyclotetraglucose syrup are shown in Table 3.

Table 1. Batch analyses of pure crystalline cyclotetraglucose

Parameters	Specifications	Analytical results				
		Lot No.				
		060901	060906	060909	01113	010524
Appearance	Odourless white crystalline powder	Passes	Passes	Passes	Passes	Passes
Identification (anthron)	Deep blue colour develops	Passes	Passes	Passes	Passes	Passes
Identification (IR)	Corresponds	Passes	Passes	Passes	Passes	Passes
Specific rotation ($[\alpha]_D^{20}$)	+240.0° – +248.0°	+244.2°	+243.1°	+244.2°	+243.6°	+244.2°
pH (10% solution)	4.5 - 6.5	5.74	5.75	5.95	5.32	5.25
Lead (atomic absorption)	≤ 1 mg/g	Passes	Passes	Passes	Passes	Passes
Moisture (Karl Fisher)	≤ 15.0%	12.7%	12.5%	12.7%	10.3%	12.8%
Residue on ignition	≤ 0.10%	0.00%	0.00%	0.01%	0.00%	0.02%
Assay	≥ 98.0%	99.6%	98.9%	99.3%	99.8%	99.3%
Chloride	≤ 0.018%	Passes	Passes	Passes	Passes	Passes
Sulfate	≤ 0.024%	Passes	Passes	Passes	Passes	Passes
Total heavy metals (as Pb)	≤ 5 ppm	Passes	Passes	Passes	Passes	Passes
Arsenic	≤ 2 ppm	Passes	Passes	Passes	Passes	Passes
Nitrogen	≤ 0.01%	Passes	Passes	Passes	Passes	Passes
Viable counts	≤ 300 cfu/g	40 cfu/g	20 cfu/g	40 cfu/g	20 cfu/g	10 cfu/g
Yeasts and molds	≤ 100 cfu/g	10 cfu/g	<10 cfu/g	10 cfu/g	<10 cfu/g	<10 cfu/g
Coliform organisms	Negative	Negative	Negative	Negative	Negative	Negative
Colour	≤ 0.100	0.029	0.034	0.034	0.051	0.046
Turbidity	≤ 0.050	0.011	0.015	0.012	0.018	0.025

Table 2. Composition of cyclotetraglucose syrup[a)]

Component	**Content**
Glucose	4.1%
Fructose	0.1%
Isomaltose	5.3%
Maltose	0.45%
Other disaccharides[b)]	2.15%
Trisaccharides[c)]	4.0%
Cyclotetraglucose	36.2%
Branched cyclotetraglucoses:	
NJ	5.5%
NK	8.5%
NM	1.5%
NO	1.6%
NP	0.9%
Other unidentified saccharides[d)]	29.7%

Abbreviations

NJ: *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→4)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}

NK: *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}

NM: *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}

NO: *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}

NP: *cyclo*-{→6)-[α-D-Glc*p*-(1→4)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}

a) Batch No. 011005 was analyzed. This batch has a dry matter content of 71.7%. The contents of the different components shown in this table refer to dry matter, i.e. they add up to 100%.

b) Other disaccharides are nigerose, trehalose, kojibiose, neotrehalose, etc.

c) Trisaccharides are isomaltotriose, panose, maltotriose, etc.

d) Other unidentified saccharides are not identified because HPLC peaks are too small.

Table 3. Batch analyses of cyclotetraglucose syrup

Items	Specifications	Analytical results				
		Lot No.				
		020828	030220	030606	40908	50201
Solid content	≥ 70.0%	72.0	72.2	72.4	72.6	72.2
Residue on ignition	≤ 0.05%	0.00	0.00	0.00	0.00	0.00
pH	4.0 - 6.5	5.2	5.6	5.8	4.9	5.7
CNN content [a)]	30.0 - 40.0%	36.4	36.8	35.7	33.2	33.8
Lead	≤ 1 ppm	< 1	< 1	< 1	< 1	< 1
Viable count	≤ 300 cfu/g	30	30	100	25	22
Yeasts and molds	≤ 100 cfu/g	0	0	0	0	0
Coliform organisms	Negative	Negative	Negative	Negative	Negative	Negative
Colour	≤ 0.100	0.021	0.024	0.043	0.030	0.017
Turbidity	≤ 0.050	0.000	0.000	0.000	0.000	0.000
Glucose (%)	—	5.0	3.1	2.2	3.3	2.9
CNN structure content [b)]	45.0 - 55.0%	52.0	51.6	50.5	49.3	48.5
Non-reducing sugar	≥ 70%	74.0	78.6	77.9	72.9	—
Heavy metals (as Pb)	≤ 5 ppm	< 5	< 5	< 5	< 5	< 5
Arsenic (as As_2O_3)	≤ 2 ppm	< 1	< 1	< 1	< 2	< 2

a) "CNN" means cyclic nigerosyl-1,6-nigerose.

b) "CNN structure" means CNN and branched CNN species.

4. 2. Possible impurities

Impurities may be brought into the production process with the applied raw material (food-grade starch), the applied enzyme preparations (crude enzyme preparation from *B. globisporus*, food-grade amylolytic enzymes) and the applied processing aids and equipment.

Residues and enzymatic break-down products of the food-grade starch are of no safety concern.

Branched derivatives of cyclotetraglucose occur in cyclotetraglucose syrup. However, these oligosaccharides will not be absorbed as such to any significant extent because of their hydrophilic nature and molecular size. They are likely to either be metabolized by the intestinal microbes or to be excreted unchanged with the feces. Their presence in cyclotetraglucose syrup should, therefore, not be of any safety concern.

By-products from the crude enzyme preparation of *B. globisporus* could arise either from the fermentation medium (which is composed entirely of food-grade ingredients) or from the metabolism of the *B. globisporus* cells. However, most of these impurities will be removed by decolouration and desalting. Since *Sporosarcina globispora* lacks a toxigenic potential, the presence of any remaining traces of such impurities in cyclotetraglucose should not be of safety concern.

Minerals that would be carried over into the final product from the raw materials or the production process are detected by the measurement of ash. The batch analyses of pure crystalline cyclotetraglucose and cyclotetraglucose syrup demonstrate that the actual ash levels are far below the specified maximum levels.

Since cyclotetraglucose is not a readily available substrate for microbial growth and since its manufacturing process includes two heat-treatment steps and further steps which would minimize microbial contamination, there appears to be no real need for specifically requesting compliance of pure crystalline cyclotetraglucose with parameters of microbiological purity. Nonetheless, three such parameters are included in the internal company specifications for quality control purposes (Table 1). In the case of cyclotetraglucose syrup which contains water and different fermentable carbohydrates, the parameters of microbial purity are included in the mandatory part of the specifications (Table 3).

5. Functional uses

5.1 Use as a carrier

Cyclotetraglucose can be used as a carrier for flavours and certain nutritive substances such as PUFAs (DHA, EPA) or certain vitamins, much like other carbohydrates such as starch, maltodextrin, nonreducing sugars (e.g., trehalose) and polyols (e.g., sorbitol) which are used for this purpose already (Oku et al., 2004).

Cyclotetraglucose has been found to be a particularly suitable carrier for certain PUFAs. However, such a carrier is usually not needed if the PUFAs are presented as a food supplement in the form of capsules. Only if the PUFAs are to be admixed to certain solid (dry) foods, as, for example, breakfast cereals and formula diets (in powder form). The use of cyclotetraglucose as a carrier for nutritive substances will not make a significant additional contribution to its total estimated daily intake.

5.2 Use as soluble dietary fiber for a nutritional purpose

Resistance to digestion and lacking absorption in the human small intestine are key criteria for classifying oligosaccharides or polysaccharides as dietary fibers. According to some definitions, isolated dietary fibers ought, in addition to non-digestibility, exhibit at least one beneficial physiological effect, such as an improved intestinal regularity, attenuated blood cholesterol levels or a blunted postprandial blood glucose response. Cyclotetraglucose corresponds to that biological definition of dietary fiber as shown by the different metabolic studies that were performed with pure crystalline cyclotetraglucose or cyclotetraglucose syrup. Whether, cyclotetraglucose would be detected as a dietary fiber by any of the official analytical methods that have been developed for fiber analyses of food, has not been investigated.

6. Reactions and Fate in Food

Cyclotetraglucose is stable under the pH and temperature conditions during the manufacturing and storage of food. Under alkaline conditions (pH 9), a slow degradation was observed (less than 10% during 5 days) (Weissenfels, 2005).

Cyclotetraglucose is hydrolyzed by isomaltodextranase (EC 3.2.1.94), but not by glucoamylase (EC 3.2.1.3), dextranase (EC 3.2.1.11), isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), salivary (human) and pancreatic α-amylases (Côté & Biely, 1994; Nishimoto, 2002).

7. References

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