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# Finding of Cyclodextrans and Attempts of their Industrialization for Cariostatic Oligosaccharides

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**Abstract: Cyclic isomaltooligosaccharides or cyclodextrans (CIs) are cyclic oligosaccharides of α-1,6 linked glucose residues. CIs are highly water-soluble and were found to strongly inhibit glucansucrase activity of mutans streptococci, so, CIs are expected to be utilized as cariostatic compounds. They are produced from dextran catalyzed by cyclic isomaltooligosaccharide glucanotransferase (CITase) and substrate dextran is produced from sucrose catalyzed by dextransucrase. CIs were found and isolated from the culture supernatant of *Bacillus circulans* T-3040 strain when it was cultured with dextran. The structure of CIs were determined by enzyme digestion test, $^{13}$C-NMR analysis, and mass spectrum analysis. In order to produce CIs for commercial scale, the high dextran producing strain *Leuconostoc* sp. S-51 was isolated and the *B. circulans* T-3040 strain was mutated to produce about 110 times as much CITase as that of wild type strain. We also successfully detected CIs in brown sugar, which suggests CIs exist in nature.**

**Key words: cyclodextran, cyclodextran glucanotransferase, dental caries, inhibitor**

Cyclodextrans (CIs) are cyclic oligosaccharides consisting of D-glucoses bound through α-(1→6) linkages produced from dextran catalyzed by cyclic isomaltooligosaccahride glucanotransferase (or cyclodextran glucanotransferase, CITase).[1)] In the beginning, CIs were expected to be a novel dominant candidate for making inclusion complexes with some larger components or highly hydrophobic components that hardly ever make a complex with cyclodextrins (CDs) because CIs are highly water-soluble and have larger cavities in the center than that of CDs. However, no significant inclusion ability comparable to that of CDs has been found in CIs.[2)] On the other hands, CIs strongly inhibit glucansucrase activity,[3)] which is thought useful in preventing dental caries, while CDs do not have this property. The cariostatic activity of CI is much stronger than that of palatinose [3)] or polyphenol.[4)] We have been doing research and development on CI production to utilize CI as a novel dental caries inhibitor.

### *Isolation of cyclodextran(CI)-producing bacterial strains and structural determination of CI.*

Dextran digestive strains were isolated from soils, and in the culture supernatant of *Bacillus circulans* T-3040 strain when it was cultivated with dextran, three unknown peaks were detected by HPLC analysis (Fig. 1).[1)] Those oligosaccharide peaks a, b and c were collected and purified. They were digested by endodextranase but not by exodextranase, suggested they were α-1,6 linked glucose residues but not linear in form. $^{13}$C-NMR and mass analyses were done on the purified oligosaccharides a, b and c. As shown in Fig. 2, the chemical shifts of $^{13}$C-NMR spectra of C-1, C-2, C-3, C-4, C-5 and C-6 of the isolated oligosaccharides indicated the same signals of glucopylanoside (6-Glc*p*) observed in α-1,6 glucan (dextran). $^{13}$C-NMR analysis also showed all the isolated oligosaccharides consisted of 6-Glc*p*.

Mass spectra analysis was also done. As shown in Fig. 3, $[M+H]^+$ ions of 1135.38, 1297.43 and 1459.48 *m*/*z* were observed in the isolated products. Molecular masses of isomaltoheptaose, isomaltooctaose and isomaltononaose are calculated as 1152, 1302 and 1476, respectively. However, molecular masses of the isolated sugars were calculated as 1134, 1296 and 1458, respectively, which are 18 (the molecular mass of one $H_2O$) smaller than those of isomaltooligosaccharides consisting of the same molecular numbers of glucose units. That is to say, the novel sugar products produced by *B. circulans* T-3040 strain were cyclic isomaltooligosaccharides (cyclodextrans, CI). We identified them as cycloisomaltoheptaose, cycloisomaltooctaose, and cycloisomaltononaose and named them CI-7, CI-8 and CI-9, respectively. In addition, larger CIs at least up to CI-17, were also observed in the CI-producing bacterial strain culture supernatant when it was grown with dextran.

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Abreviations: CI, cyclodextran; CITase, cyclic isomaltooligosaccharide glucanotransferase, cyclodextran glucanotransferase; CD, cyclodextrin; GTF, glucosyltransferase; NTG, *N*-methyl-*N*′-nitro-*N*-nitrothoguanidine

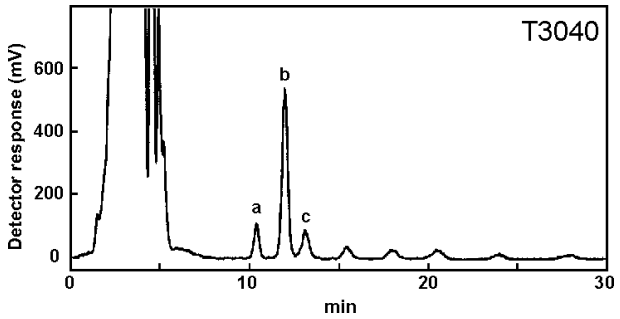


**Fig. 1.** Elution pattern of cyclic oligosaccharides produced by *Bacillus circulans* T-3040.

Culture supernatant cultivated with 2% dextran 40 of *B. circulans*, T-3040 was taken and the same amounts of acetonitrile was added; 10 μL was separated by HPLC with TSK-gel Amide 80 column (ϕ4.6 mm×25 cm, Tosoh) with 55% acetonitrile with a flow rate of 1 mL/min. Peaks of a, b and c were observed at the retention times of 10.6, 12.1 and 13.4 min, respectively.

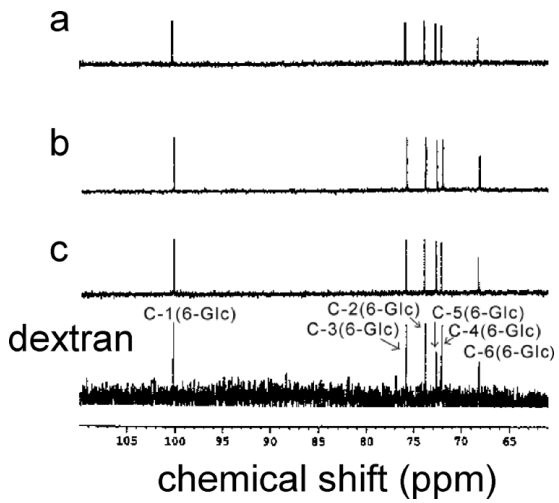


**Fig. 2.** $^{13}$C-NMR spectra of the peaks, a, b, c.

Cyclic oligosaccharides of a, b and c produced by *B. circulans* T-3040 eluted by HPLC in Fig. 1 were collected and lyophilized. The $^{13}$C-NMR spectra of the oligosaccharides samples were measured with a Bruker DRX 600 spectrometer with a detector of X ($^{15}$N, $^{13}$C, $^{31}$P)-{$^{1}$H} QNP probehead with complete decoupling at 298 K and the Larmor frequency was 150.90 MHz.

### *Inhibition of glucan synthesis of glucosyltransferase (GTF) from Streptococcus mutans by CI.*

Water-insoluble glucan produced by *Streptococcus mutans* and/or *S. sobrinus* forms dental plaque to cause dental caries. CIs are composed of α-1,6 glucosidic linkages which is the same as those of dextran. However, all the C-6 position of CIs are covalently linked to the C-1 of the adjacent glucosyl residue, while one C-6 position of the dextran molecule is free. Based on these characteristics of CIs, they are expected to inhibit enzymatic synthesis of dextran and mutan.[3] The scheme of preventing dental caries by CI is indicated as Fig. 4. First, mutans streptococci produce water-soluble glucan from sucrose, then dental plaque is formed and acid is released to cause dental caries. CI inhibit the first reaction of producing glucan from sucrose. Water-insoluble glucan (mutan) synthesis by the glucosyltransferase (GTF) reaction from *Streptococcus mutans* IFO 13955 with and without CI was measured. As shown in Fig. 5, all the CI (2 mM each) from CI-7 to CI-12 inhibited GTF. The level of GTF inhibition was not

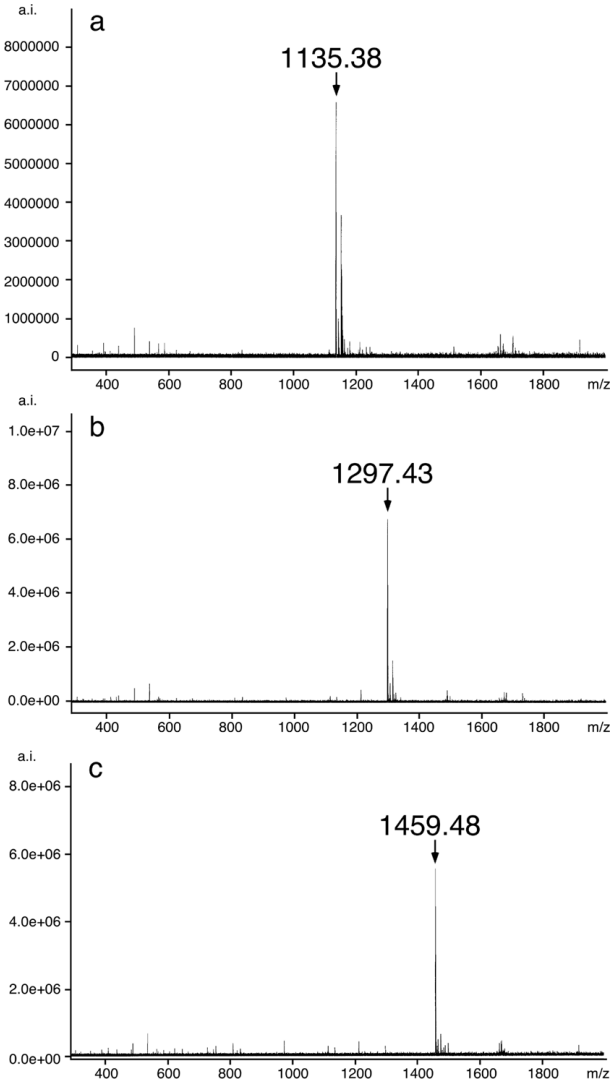


**Fig. 3.** Mass analysis of the peaks, a, b and c.

Mass analysis was performed on an ApexII (7 tesla) Fourier-transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, MA, USA). Each freeze dried a, b and c sample was diluted by 50% methanol containing 2% acetic acid and was infused by a syringe pump through the Analytica ESI source at a flow rate of 0.1 mL h-1. Parameters were tuned by each sample to observed a spectrum of good intensity, and the mass spectrometer was calibrated with fragment ions of angiotensin I in the positive-ion mode.

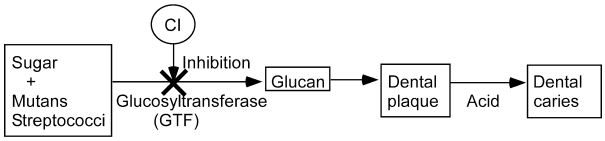


**Fig. 4.** Pathway of dental caries formation and cariostatic function of CI.

significantly changed by the difference of molecular sizes. The mixed CI samples (0.2%) also well inhibited GTF and almost no mutan was produced. When CI was given to rats, less than 0.2% CI could reduce their percentages of suffering from dental caries caused by *Streptococcus mutans* or *S. sobrinus*, of which effects were stronger than that of polyphenol.[4] CI strongly decreases glucansucrase activity by competitive inhibition, with $K_i$ values of 0.2 to 0.6 mM, whereas the $K_m$ value of glucansucrase for a substrate (sucrose) was 24 mM, suggesting that CI might prevent dental caries when used as a cariostatic sub-

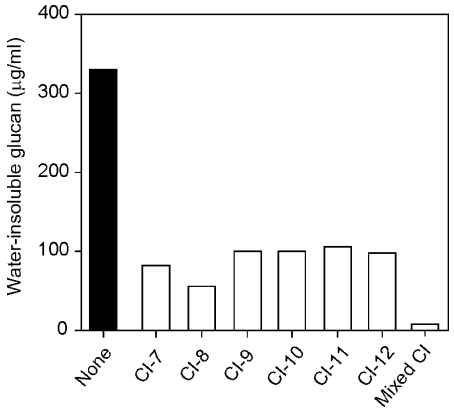


**Fig. 5.** Inhibition of water-insoluble glucan production by CIs.

GTF (0.2 U) from *S. mutans* was mixed with 5% sucrose and with 2 mM CI-7, CI-8, CI-9, CI-10, CI-11 or CI-12 or 0.2% CI mixture at pH 5.5 as described before.[3] After the incubation at 37°C for 18 h, produced water-insoluble glucan was collected by centrifugation, washed with water, dissolved in 1 M NaOH, and total sugar was measured by the phenol-sulfuric acid method.[10]

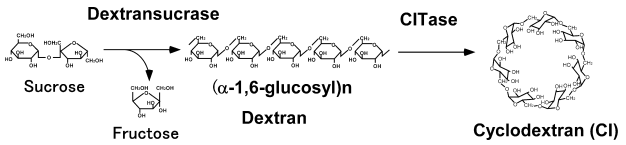


**Fig. 6.** Enzymatic synthesis of CI from sucrose.

stance.[3]

***Difficulties for production of CI for commercial use.***

CI is produced from dextran catalyzed by CITase and the substrate dextran is produced from sucrose catalyzed by dextransucrase (Fig. 6). To produce CI for commercial use, there were four difficulties. First, sucrose is expensive for a food resource; second, a high dextran-producing bacterial strain is needed; third, CITase activity of the bacterial strains are very low for CI production, and fourth, as CI is recognized as a novel compound, it should be proved to be safe.

To solve the first problem, we tried to use sugar cane extract and waste molasses. With sugarcane extract, dextran-producing bacterial strains grew well and large amouts of dextran were produced. We have decided to use sugarcane extract as a sucrose resource instead of using refined sugar.

***Isolation of high dextran-producing strain for CI production.***

We isolated a well-producing strain for dextran containing abundant α-1,6-D-linkages, and also analyzed the properties of its glucan and glucansucrase. Nine kinds of glucan-producing strains in their culture supernatant were isolated from the lines of a sugar-manufacturing factory (Fig. 7). Eight strains out of them produced water-soluble glucan. $^{13}$C-NMR analysis suggested that the five out of nine of glucans mainly consisted of 6-Glc*p* and the other four glucans were branched. Among the 6-Glc*p* glucan-producing strains, S-51 was selected because of its extracellular glucansucrase activity and the rate of CI conversion by CITase was highest, being twice as much as *L. mesenteroides* NRRL B-512F, which is the most commonly used dextran-producing strain.[5]

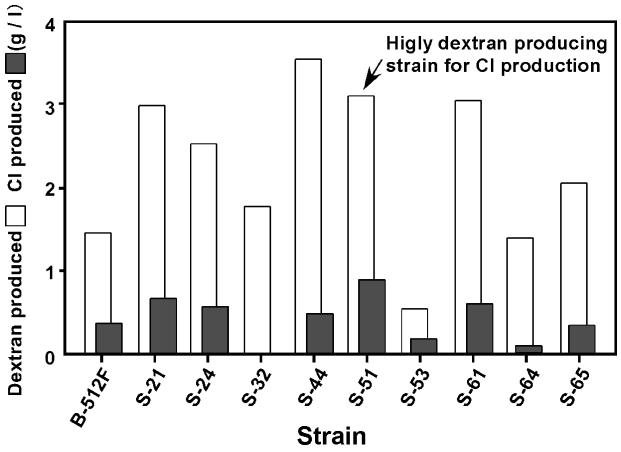


**Fig. 7.** Isolation of glucan-producing strains and CI production from their glucan.

Microorganisms that form glutinous colonies on 2%-sucrose-containing solid medium were isolated.[5] Isolated colonies were cultured with 2% sucrose at 30°C overnight. Cells were discarded by centrifugation and 50% ethanol was added to supernatants. Strains of those culture supernatants forming white precipitates by addition of 50% ethanol were isolated as glucan-producing strains. Enzyme solution (*B. circulans* T-3040 bacterial culture supernatant grown with 0.5% dextran) (50 μL) was added to an equal volume of 2% (w/v) of each dextran in 80 mM Na-acetate buffer (pH 5.5). The reaction mixtures were incubated at 40°C for 2 h. CI synthesis activity was determined by measuring liberated CIs (sum of the amounts of CI-7, CI-8 and CI-9) by HPLC with a TSKgel Amide-80 column as described before.[11]

***Development of CITase activity of B. circulans T-3040 strain by mutagenesis.***

The *B. circulans* T-3040 strain was isolated from soil as a CI- and CITase-producing strain. However, its CITase activity is not strong enough for commercial production of CI. We have succeeded in obtaining a CITase gene and producing a large amount of *Escherichia coli*- recombinant CITase,[6] but it is not favorable to use an *E. coli*-recombinant enzyme for food. So, we tried to obtain a high CITase-producing strain by selecting chemical substance-resistant mutant strains. At first, *B. circulans* T-3040 was treated with *N*-methyl-*N*′-nitro-*N*-nitrothoguanidine (NTG) and NTG-resistant colonies were picked up. This treatment was repeated and when CITase activity reached about 44 times as much as that of wild type *B. circulans* T-3040 strain, its productivity did not increase any more with further NTG treatment. Kurokawa *et al*. reported that streptomycin resistance enhanced α-amylase activity in *Bacillus subtilis* 168.[7] Then, streptomycin treatment was done repeatedly on the NTG-resistant mutant from *B. circulans* T-3040 and obtained about 110 times as much CITase-producing mutant strain as the wild type strain (Fig. 8).[8] That is enough for producing large amounts of CIs on a commercial scale.

***Safety test for CI and isolation of CI from brown sugar.***

To use CIs for food, it is necessary that CIs are safe. An acute toxicity test and sub-acute toxicity test were done on CIs with rats and no toxicity was detected. Then, we tried to find CIs in traditional food to prove CI is a

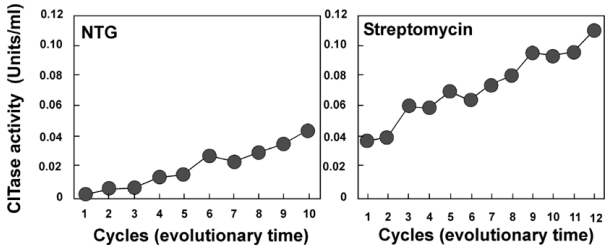


**Fig. 8.** CITase activity of the mutant strains from *B. circulans* T-3040.

*B. circulans* T-3040 strain was first treated with NTG and then streptomycin as described before.[8] NTG- or streptomycin-resistant cells were cultured with Blue dextran 2000 (Amersham Biosciences) containing solid culture. Colonies that made halos were picked up and cultured with 2% dextran 40 (Amersham Biosciences)-containing culture and CITase activity of the culture supernatants was measured as described in the legend of Fig. 7.

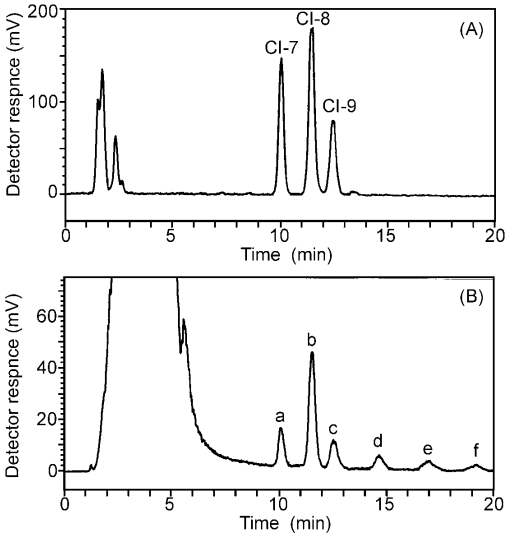


**Fig. 9.** High performance liquid chromatograms of brown sugar extract produced by Iriomote Sugar Factory.

The standard CI-7, CI-8 and CI-9 were prepared as described in the text. The brown sugar sample (1 kg) was dissolved in water and insoluble compounds were removed by centrifugation. Water-soluble oligosaccharides containing supernatant fraction was subjected to charcoal column, washed with water, eluted with 50% ethanol, and concentrated with a vacuum evaporator. The sample was subjected to a HP-20 column, washed with water, eluted with 20% ethanol, and concentrated with a vacuum evaporator again. Linear oligosaccharides and remaining glucans were digested to glucose with highly branched-dextran glucodextranase,[12] subjected to a Sep-Pak Vac 20 cc (5 g) C18 cartridge (Waters), washed with water and eluted by 20% ethanol as described before.[9] The sample was concentrated with a vacuum evaporator. Sample was dissolved in appropriate amounts of 50% acetonitrile and the remaining oligosaccharides were analyzed by HPLC as described in the legend of Fig. 1. (A) Standard CI-7, CI-8 and CI-9 samples. (B) Kokuto produced by Iriomote Sugar Factory.

natural compound. Several different brands of brown sugar were extracted by ethanol. Sucrose, reducing sugars, or polysaccahrides were removed by charcoal column chromatography. Remaining linear oligomers were digested to glucose with exoglucanase, and that glucose was removed by a Sep-Pak C18 cartridge (Amersham Biosciences). Then remaining oligosaccharides were measured by HPLC. As shown in Fig. 9, the peaks eluted at the same retention times as standards CI-7, CI-8 and CI-9 were detected. When analysis was done on these peaks, the signals indicating the same molecular mass of CI-7, CI-8 and CI-9 was detected.[9] We successfully detected CIs in one of the traditional foods, brown sugar. CIs must have been eaten by human beings for a long time. Now we are trying to do the safety and cariostatic function tests for CIs in humans.

### *Trial production of CI.*

Using the high dextran-producing strain *Leuconostoc* sp. S-51 and high CITase-producing mutant strain *B. circulans* G22-10,[8] we tried to produce CIs on a large scale and a glucan-inhibition test was done on the produced CI samples. Making highly purified CIs costs a lot. However, lower purified samples contain some remaining glucan and isomaltooligosaccharides or other impurities. Small-sized isomaltooligosaccharides such as isomaltose also inhibit glucansucrase activity, but other components can activate GTF. The content of the sum of CI-7, CI-8 and CI-9 in the CI samples was between 11 and 16% and all of them contained small amounts of larger-sized isomaltooligosaccharides and sugar polymers, but all CI samples inhibited water-insoluble glucan production by *S. sobrinus* and *S. mutans* more effectively than the same amounts of polyphenol. Now we are trying to produce CIs on a commercial level, and to see what type of food is suitable to use this novel, unique anti-dental caries cyclic ololigosaccharide, CI.

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## 抗う蝕性環状オリゴ糖・サイクロデキストランの発見から実用化技術開発へ

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サイクロデキストラン(CI)はグルコース分子がα-1,6結合で連結した環状イソマルトオリゴ糖である．CIは環状イソマルトオリゴ糖グルカノトランスフェラーゼ(サイクロデキストラングルカノトランスフェラーゼ，CITase)によってデキストランより合成され，基質のデキストランはデキストランスクラーゼによりスクロースから合成される．CIは*Bacillus circulans* T-3040菌株を，デキストランを含む培地で液体培養した培養上清に発見され，$^{13}$C NMR分析および質量分析により構造決定された．CIはCDよりもきわめて水溶性が高く，抗う蝕性能を有する．この機能を利用して，虫歯を予防するオリゴ糖としてCIを実用化する目的で，製糖工場内よりα-1,6結合の割合の高いCI生産に適したデキストランを多量に短時間で生産する菌株取得に成功し，薬剤耐性菌の選抜によるCITase高生産変異株の取得を試み，約110倍にCITase活性が上昇した変異株の取得に成功し，CI生産レベルを著しく高めた．さらに，黒糖中のオリゴ糖成分を抽出，HPLC分析した結果，CIと全く同じリテンションタイムに溶出するピークが観察され，質量分析によりCI-7，CI-8，およびCI-9と同じ分子量を示すシグナルが確認でき，CIは黒糖中に含まれる天然オリゴ糖であることが示唆された．

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〔質問〕　江崎グリコ　栗木

1) *Streptococcus mutans* あるいは *Streptococcus sobrinus* を用いたバイオフィルムの合成阻害活性．2) 上記菌株由来のGlycosyltransferaseの阻害活性でCIの抗う蝕性を示されています．一方，口腔内でプラークを形成する微生物は，これら2種の菌株に限らず，非常に多種多様なものであるということがわかっています．CIを抗う蝕性を証明するために安全性等が確保できた，できる限り早い段階で，ヒト口腔内での試験をすることをお奨めいたします．

〔答〕

有益なアドバイスどうもありがとうございます．現在ヒトでの評価試験を行うために必要なサイクロデキストランサンプルを調整し終えた段階です．来年の早い時期にヒト試験をする予定です．製品化するのは試験がすべて終わってからと考えています．

〔質問〕　石川県立大　谷口

1)　デキストランからのCI (混合物) の収率はどれくらいですか．2)　実用的のターゲットとして虫歯予防(CI-7～9) を重点とするのか，包接化合物 (CI10) を目指すのですか．

〔答〕

1)　CI-7からCI-12までを有効成分と考えた場合，収率は50% 程度です．デキストランの原料のスクロースからと考えると，さらにこの半分になります．2) まず，虫歯予防素材としての実用化を目指す予定です．包接剤として用いるためにはまだ検討しなければならない事項が多くありますので，次の段階と考えています．