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Effect of Antimicrobial Activity by Chitosan Oligosaccharide N-Conjugated with Asparagine

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Abstract Since the number of amino groups which are exposed by deacetylation of acetyl-D-glucosamine influences antimicrobial activity, a chitosan oligosaccharide (COS) derivative by N-conjugation of COS with asparagine, an amino acid with two amino groups, was synthesized and the antimicrobial effect on *E. coli* growth was compared with other COS derivatives which were N-conjugated with glycine, alanine, aspartic acid, cysteine, and methionine, and unmodified COS. The structure of asparagine N-conjugated COS (Asn-COS) derivative was identified by using a FT-IR, ^{13}C FT-NMR, and an elemental analyzer. The antimicrobial activity of Asn-COS against *E. coli* growth was significantly improved as compared to the other COS derivatives as well as COS itself. This means that Asn-COS with two positive charges strongly interacts with the carboxyl negative charges on the bacteria cell wall. The results for Asn-COS were as follows: 100% bactericidal activity, 0.002% MIC, and no growth of *E. coli* during 3 days of culture time, suggesting that Asn-COS may be useful as a new antibiotic agent.

Key words: Chitosan oligosaccharide, derivative, antimicrobial, asparagine, conjugation

Chitin, a polymer of *N*-acetylglucosamine (β-1,4 linked 2-acetamido-D-glucose), is a cellulose-like biopolymer present in the exoskeleton of crustaceans and can be obtained from the shell waste of the crab, shrimp, and crawfish processing industries. Chitosan is derived from chitin by deacetylation [8], and exhibits a wide variety of physiological activities including an antitumor effect [21-23], immuno-stimulating effect [16], antibacterial effect [3, 9, 18], and cholesterol-reducing effect [14, 20]. In spite of the various functional properlies of chitosan, its high molecular weight and high viscosity might restrict the uses *in vivo*. Therefore, an attention in chitosan fields has recently been increased to the production of useful chitosan digosacchgrides. This fact has led to the development of a variety of the related chitosan enzymes [1, 11-14, 26]. Many reports [9, 22-24] already showed that particular chitosan oligosaccharides are more available in their physiological properties. In addition, the antibacterial effect of chitosan is closely related to its molecular weight and degree of acetylation [3, 9]. Chitosan, unlike chitin, possesses primary amino groups in its structure and the number of these amino groups is related to the rate of antibacterial activity. Allan and Hadwiger [2] reported that the positively charged amino groups in chitosan inhibit the growth of fungi or microbacteria through the polyelectrolyte complexes with negatively-charged carboxyl anion groups present in their cell walls. This probably indicates that the higher the number of amino groups, the higher the antibacterial activity.

Therefore, in this study, a chitosan oligosaccharide (COS) derivative which was N-conjugated with asparagine (Asn-COS) was synthesized. Asparagine has two amino functional groups on its backbone and side chain. The current authors have previously prepared various COSs using a dual bioreactor membrane system and examined their antibacterial activities [4-6].

The objective of this study was to synthesize an N-conjugated COS derivative with multi-amino groups through the introduction of asparagine and observe the change in the antimicrobial activity of synthesized Asn-COS on the growth of *E. coli*. And the activity was compared with other COS derivatives N-conjugated with glycine, alanine, aspartic acid, cysteine, and methionine as well as unmodified COS.

Materials and Methods

Materials and Apparatus

The chitosan (degree of deacetylation, 89%; viscosity 20 cps), used as a starting material for the preparation of COS,

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was donated from Kitto Life Co. (Korea). The chitosanase (694 units per 1 g protein, derived from the *Bacillus pumilus* BN-262 strain; molecular weight, approximately 30,000 Da; optimal pH and temperature, 5.5-6.5 and 30-50°C, respectively), a specific enzyme for the hydrolysis of chitosan, was purchased from Wako Pure Chemical Co. (Osaka, Japan). D-Glucosamine, a repeat unit of chitosan, was obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

For the syntheses of COS derivatives N-conjugated with glycine (Gly), alanine (Ala), aspartic acid (Asp), cysteine (Cys), and methionine (Met) as well as asparagine (Asn), their N-t-tert-butyloxycarbonyl (Boc) amino acid derivatives, Boc-Gly, Boc-Ala, Boc-Asp β-benzylester, Boc-S-(p-methoxybenzyl)-L-Cys, Boc-L-Met, and Boc-Asn, were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). N,N'-Dicyclohexylcarbodiimide (DCC), triethyl amine (TEA), and trifluoroacetic acid (TFA) were also from Sigma Chemical Co. (St. Louis, U.S.A.). All the other chemicals were of analytical grade.

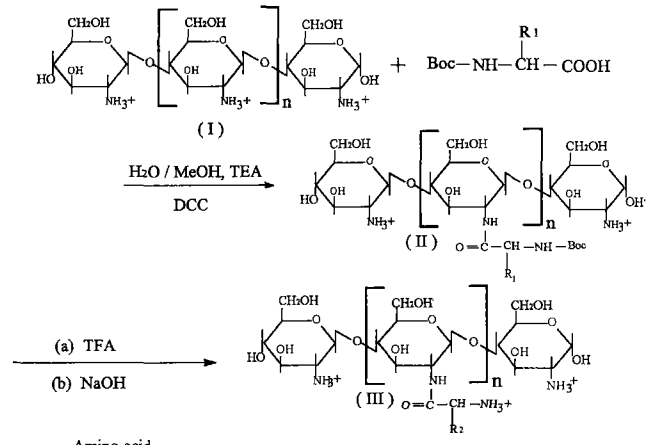
The apparatus used in this study included a UV spectrophotometer (Hitachi U-3210, Japan), FT-IR spectrometer (Perkin Elmer Spectrum 2000, Branchburg, U.S.A.), FT-NMR (Varian Unity Plus 300, Varian Co., U.S.A.), elemental analyzer (Perkin Elmer optima 3300 XL, Branchburg, NJ, U.S.A.), and a Micro Acilyzer (EPMA 1600, Shimadzu, Tokyo, Japan) for desalting.

Preparation of COS

The COS was prepared from chitosan by using chitosanase in a dual bioreactor system according to the previously reported method [5] and fractionated into oligomers smaller than 10,000 Da using a molecular weight cut-off (MWCO) 10 kDa ultrafiltration membrane. The COS recovered was lyophilized on a freezing drier for 5 days.

Synthesis of Amino Acid N-Conjugated-COS (AA-COS) Derivatives

The COS derivatives were synthesized by the N-conjugation of the Boc amino acid (Boc-AA) derivatives to the C-2 position of the glucosamine monomer in the COS, according to the synthetic scheme shown in Fig. 1. The COS (0.25 g, 1 mmol based on the free amino group in the COS) dissolved in 50 ml of distilled water was added to 200 ml of methanol and adjusted to pH 6.8 with TEA. Boc-AA (10-200 mmol) with an equivalent DCC was added to the mixture, which was then shaken for 1-24 h. After the reaction was over, the resulting reactant was filtrated to remove any DCU (dicyclohexylurea) conversed from the DCC, concentrated, and then kept at 2°C overnight. The resulting precipitate, Boc-AA-COS, from the reactant was obtained by the addition of an adequate amount of ether, and then lyophilized after filtration. In order to remove the Boc group from the product, it was treated with 20 ml of TFA for 6 h, neutralized with 4 N NaOH, desalted with a



	Amino acid of AA-COS	R1	R2	II → III
1	glycine	H	H	(a)
2	alanine	CH_3	CH_3	(a)
3*	aspartic acid	$CH_2-\overset{O}{\overset{\|}{C}}-O-\bigcirc$	$CH_2-\overset{O}{\overset{\|}{C}}-OH$	(a), (b)
4	asparagine	$CH_2-\overset{O}{\overset{\|}{C}}-NH_3^+$	$CH_2-\overset{O}{\overset{\|}{C}}-NH_3^+$	(a)
5*	cysteine	$CH_2-S-\bigcirc-OCH_3$	CH_2-SH	(a), (b)
6	methionine	$CH_2CH_2-S-CH_3$	$CH_2CH_2-S-CH_3$	(a)

Fig. 1. Synthetic scheme of chitosan oligosaccharide derivatives (AA-COS) N-conjugated with different amino acids.

Micro Acilyzer, and then concentrated. The final product, an amino acid-conjugated COS derivative (AA-COS), was obtained by precipitation with acetone, filtration, and lyophilization. For Boc-AA derivatives with side functional groups, such as Boc-Asp β-benzyl ester and Boc-S-(p-methoxy-benzyl)-Cys, the products, after being treated with TFA, were incubated with 20 ml of 6 N NaOH for 12 h at room temperature, and the remaining procedures were similary to the method described above.

The chemical structure of the COS derivatives was determined by the FT-IR spectrum, ^{13}C FT-NMR spectrometer, and elemental analysis.

Antimicrobial Activity

The antimicrobial activity of the COS derivatives on the growth of *E. coli* was examined. A mixture of 0.5 ml of the cultured bacteria solution (the number of colony was ranged from 200 to 300 after 24 h incubation), 0.5 ml of the autoclaved sample solution, and 49 ml of a 0.05 M acetate buffer (pH 6.0) was incubated with shaking at 37°C for 1 h or 2 h. One ml of the mixture was then diluted 10-fold with tryptic soy broth and added to a tryptic soy agar (TSA: Difco) medium, which was placed in a petri-dish and incubated at 37°C for 24 h. After incubation, the colonies formed were counted to examine the bactericidal activity, which was calculated using the following formula: Bactericidal activity (%)=[(C-T)/C]×100, where C is the number of colonies counted in the control and T is that in the tested sample plate.

The minimum inhibitory concentration (MIC) was tested using a two-fold serial broth dilution as follows: *E. coli* (10^5-10^6 CFU/ml) was inoculated in 5 ml of tryptic soy broth which contained 1 ml of the test sample in a test tube and incubated under the same conditions as described above. The MIC was defined as the lowest concentration of the tested sample at which no cell growth was visible with the naked eye or using microscopy.

RESULTS

N-Conjugated COS Derivatives

For the preparation of effective N-conjugation of COS with an amino acid, first, the amount of the amino acid added and the reaction time were examined, empolying the N-conjugation of COS (1 mmol, based on free amino group, 0.25 g) with Boc-Gly and DCC (1-20 mmol) under the reaction conditions described above. The reaction rate was determined by the amount of amino groups remaining on the COS after the reaction, using the ninhydrin method according to Hirano *et al.* [4]. As shown in Fig. 2, the increase of Boc-Gly up to 5 mmol sharply accelerated the reaction rate. The effective reaction time was observed as 4 h (Fig. 3).

Following the results shown in Figs. 2 and 3, the asparagine N-conjugated COS derivative was prepared using Boc-Asn (5 mmol) and COS (1 mmol) with stirring for 4 h, then the chemical structure was characterized using its FT-IR spectrum, ^{13}C FT-NMR spectrometery, and elemental analysis (Fig. 4). In the FT-IR spectrum, Asn-COS showed two strong absorptions for amide bond I at

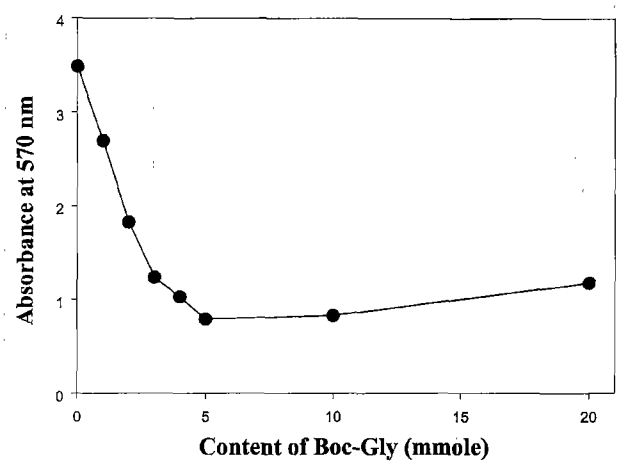


Fig. 2. Change in amount of free amino group in COS during N-conjugation of COS with Boc-Gly.
COS (2.5 g, 1 mmol) was added to 5 ml of water and 10 ml of methanol, and then mixed with Boc-Gly and DCC (1-20 mmol) in 10 ml methanol. After keeping at room temperature for 24 h, an aliquot (0.5 ml) of the mixture was tested by the ninhydrin method to determine the amount of the remaining free amino group in the COS.

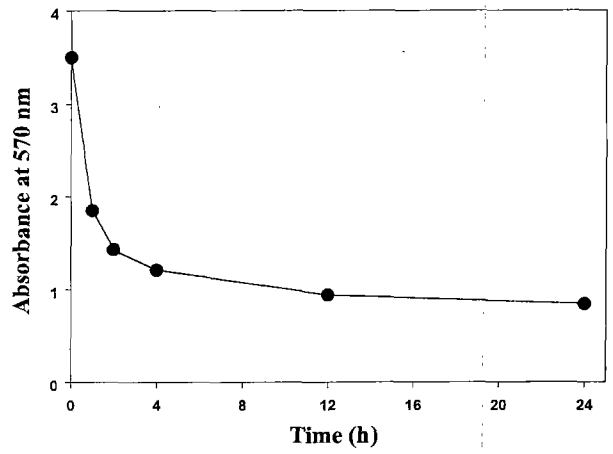


Fig. 3. Effect of incubation time in N-conjugation of Boc-Gly (5 mmol) with free amino group of COS (1 mmol).

1650-1660 cm^{-1} and amide bond II at 1550-1560 cm^{-1}, both of which indicate N-conjugation on C=O at C-2; however, no absorption at 1750 cm^{-1} for a carboxyl bond peak at the C-6 position of glucosamine was exhibited. This result indicates that asparagine was selectively conjugated only at the C-2 position of the glucosamine. An indentification of Asn-COS (10% (w/v) in D_2O solvent) in FT-NMR analysis was carried out at 45°C using 20,000 scans. The ^{13}C NMR spectrum showed a pyranose peak at around 60-110 ppm and -CO- peak by amide bonding at 178 ppm. In addition, two peaks, -CH- and -CH_2- on the side chain of asparagine, were observed at 38 ppm and 24 ppm (Fig. 4). The degree of substitution (DS) for the asparagyl group N-conjugated at the C-2 position of the glucosamine was 0.69, shown by an elemental analysis [Anal. Calc. for $C_6H_{12}NO_5(H)_{0.31}(C_4H_7N_2O_2)_{0.69}$: C, 27.94; H, 4.81; N, 5.43; ($C_4H_7N_2O_2$), 30.91. Found: C, 27.65; H, 4.93; N, 5.91; ($C_4H_7N_2O_2$), 30.01]. The DS of Asn-COS using the ninhydrin method was 0.73. The DS for the other derivatives, Gly-, Ala-, Asp-, Cys-, and Met-COS was 0.82, 0.73, 0.75, 0.85, and 0.83, respectively.

Antimicrobial Effect of Asn-COS

The antimicrobial effect of Asn-COS was investigated following its bactericidal activity against the growth of *E. coli* and compared with other AA-COS derivatives. As shown in Table 1, Asn-COS completely inhibited the growth of *E. coli* independent of the inoculation time, 1 h or 2 h. The bactericidal activity of the unmodified COSs was 59.9% and 83.3% for 1 h and 2 h of inoculation time, respectively. Regarding the bactericidal activity of the other AA-COS derivatives, most of the other AA-COS derivatives, Gly-, Ala-, Asp-, Cys-, and Met-COS, exhibited a lower activity less than 50%, except for 2 h of inoculation time with Cys-COS (61.1%). The MIC concentrations also showed a similar trend to the bactericidal activity (Table 2). Asn-

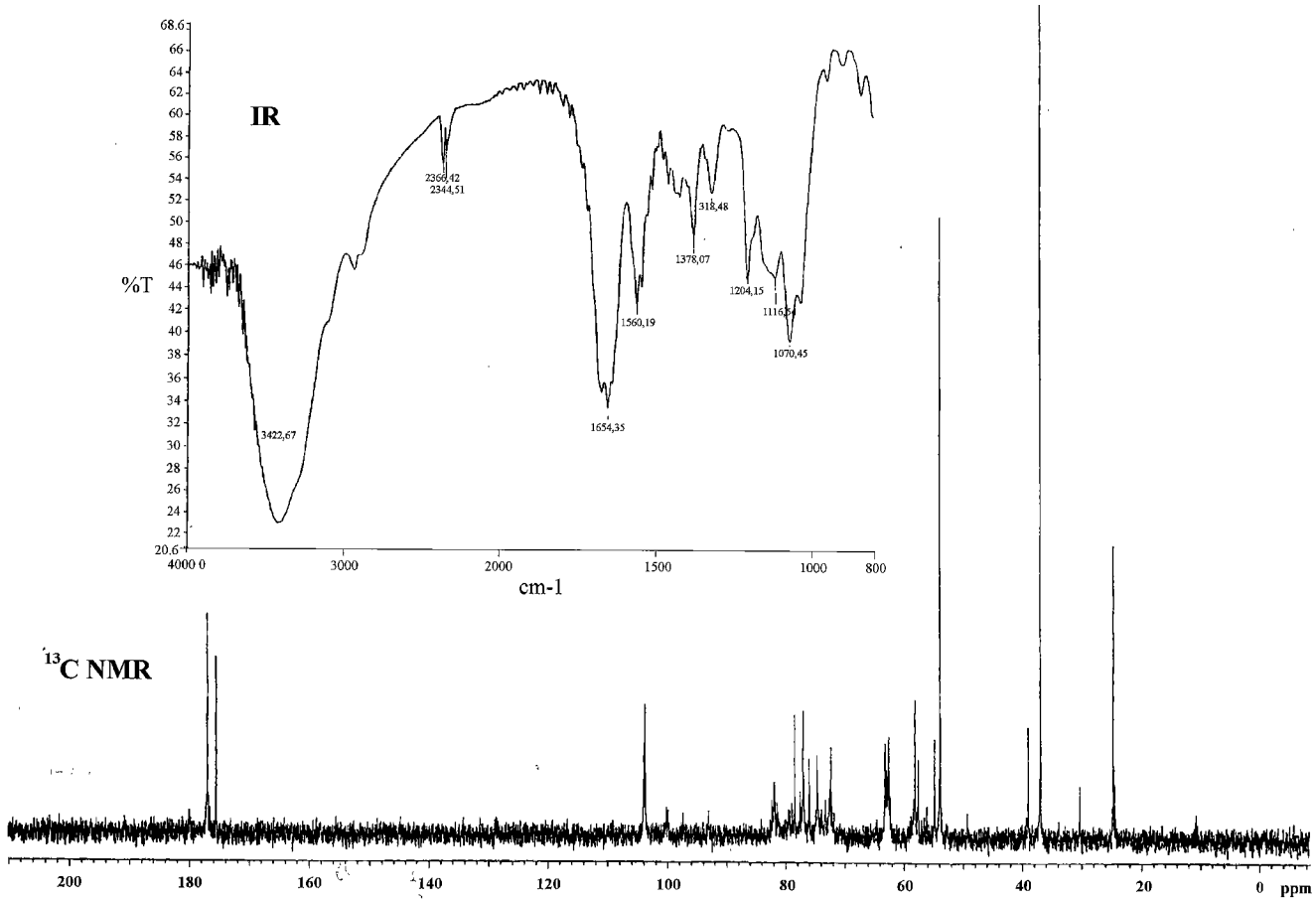


Fig. 4. The IR and ^{13}C NMR spectra of the Asn-COS derivative.

COS indicated an extremely low MIC concentration (0.002%), whereas COS was 0.063% and the other derivatives were almost ineffective. The antimicrobial effect of Asn-COS was also examined as a function of the culture time up to 3 days in relation to its concentration. Asn-COS at 0.01% or more concentration almost completely inhibited the growth of *E. coli* during 3 days of culture time (Fig. 5).

Table 1. Antimicrobial activities of COS and AA-COS against *E. coli*.

Compound	Bactericidal activity (%) by inoculation time[1]	
	1 h	2 h
COS	59.9	83.3
Asn-COS	100.0	100.0
Gly-COS	0.0	13.5
Ala-COS	4.0	8.6
Asp-COS	15.3	28.1
Cys-COS	23.7	61.1
Met-COS	13.0	23.8

[1]The experiment for antimicrobial activity was carried out using the colony count method. *E. coli* was incubated at 37°C for 24 h after 1 h or 2 h of inoculation time with a 0.1% sample concentration.

DISCUSSION

COSs as well as chitosan have been shown to inhibit the growth of several fungi and bacteria, especially pathogens [7, 10, 25], and their antimicrobial activity significantly depends on the degree of polymerization and the level of deacetylation [8, 18]. Therefore, the relationship between the activity and the level of deacetylation suggests that the number of amino groups present in the chitosan structure

Table 2. MIC of COS and AA-COS derivatives against *E. coli*.

Compound	MIC (%)[1]
COS	0.063
Asn-COS	0.002
Gly-COS	(1.0)[2]
Ala-COS	(1.0)
Asp-COS	(1.0)
Cys-COS	(1.0)
Met-COS	(1.0)

[1]The bacterial formation was determined by examination with the naked eye or microscopy.
[2]Ineffective at a concentration of 1.0% (w/v)

influence its inhibitory activity against the growth of fungi and bacteria. This is most likely due to interaction of the positive charges of the amino group at the C-2 in the glucosamine monomer in chitosan with the negative charges of carboxylic acid in the cell wall. Accordingly, the modification of the amino group at the C-2 position in glucosamine, especially adding amino groups, would enhance the antibacterial activity.

Nevertheless, only a few studies on the antibacterial effect of modified chitosan or its oligomer at C-2 have been reported. Muraki and Aiba [16] revealed that partially N-lauroyl (PNL)-chitooligosaccharides (DP 7-8) with a degree of N-lauroylation at about 50% has a fairly strong antibacterial activity against the growth of *E. coli*. Sudharshan *et al.* [20] also reported that water-soluble chitosans, such as chitosan lactate and chitosan hydroglutamate, show bactericidal activities against both Gram-positive and Gram-negative bacteria in the range of a one to five log cycle reduction within 1 h. However, these studies did not include the effect of increasing the number of amino groups at the C-2 position.

In the current study, an attempt was made to increase the number of amino groups by introducing asparagine to the C-2 of the glucosamine, thus obtaining a COS derivative N-conjugated with asparagine (Asn-COS) containing two amino groups. The antimicrobial effect of Asn-COS was then compared to that of COS and COS derivatives N-conjugated with different amino acids, such as glycine, alanine, aspartic acid, cysteine, and methionine. As a result, totally different antimicrobial abilities in both bactericidal activities and MIC concentrations were observed between Asn-COS and the other COS derivatives, including COS itself. The introduction of asparagine to COS significantly

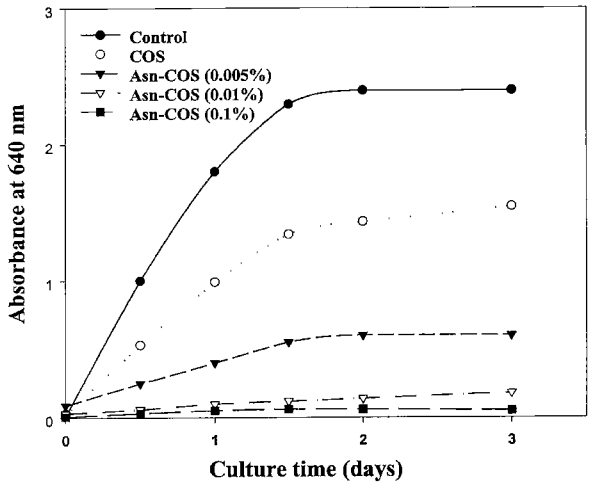


Fig. 5. Effect of Asn-COS concentration against growth of *E. coli*.
The determination of the growth of *E. coli* was estimated by its UV absorbance at 640 nm.

improved the bactericidal activity and MIC, whereas the other COS derivatives were significantly inferior to the unmodified COS in both examinations. This fact strongly suggests that Asn-COS with two positive charges strongly interacts with the carboxyl negative charges on the bacteria cell wall. The reason that the remaining derivatives showed lower activities might be because the amino groups at the C-2 of the glucosamine were blocked, even though the derivatives possessed a new amino group through the introduction of an amino acid.

All the results observed by Asn-COS were 100% bactericidal activity, 0.002% MIC, and absolutely no growth of *E. coli* during 3 days of culture time. These results imply Asn-COS will be useful as a potential new antibiotic if it is nontoxic in the body system.

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