Cosmeceutical Properties of Fructan (Levan) Produced by *Zymomonas mobilis*

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Summary

Fructan, a polysaccharide existing in plants or produced by microorganisms, is a sugar polymer of fructose with β-2,6 linkages.

In this study, we investigated some cosmeceutical properties of Fructan such as moisturizing effect, cell proliferation effect, anti-inflammation effect and cell cytotoxicity. *Zymomonas mobilis*, a microorganism producing Fructan, was cultured in a medium containing 10% sucrose and 2% yeast extract as main components for 24 hours at 37℃ and pH 7. Fructan was obtained by precipitation from the cultured medium by adding alcohol (alcohol ratio of 1:3) after removing the enzyme by centrifuging.

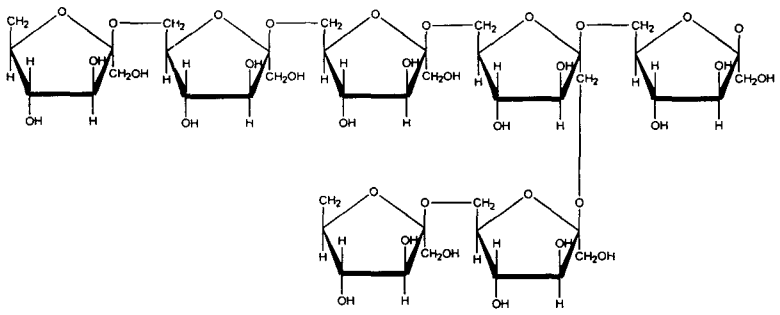
Fructan exhibited almost same moisturizing effect as hyaluronic acid and cell proliferation effect on human fibroblast and keratinocyte as well. Moreover, on cell proliferation test on bio-artificial skin constructed by 3-dimensional(3-D) culture after inducing primary skin inflammation with 0.5% sodium lauryl sulfate (SLS), the 3-D artificial skin treated with 0.01mg/ml, 0.05mg/ml of Fructan

exhibited higher cell proliferation than the 3-D artificial skin treated with SLS only. On anti-inflammation test on 3-D artificial skin evaluated by measuring secreted quantity of interleukin-1α (IL-1α) which is a pre-inflammatory mediator induced by SLS, the quantity of IL-1α on the 3-D artificial skin treated with 0.01mg/ml, 0.05mg/ml of Fructan was less than the one on the 3-D artificial skin treated with SLS only. As a result of these studies, Fructan has anti-inflammation effect against inflammatory reaction by a skin irritant as well as cell proliferation effect in bio-artificial skin. Fructan was also evaluated as a safe material without any toxicity in safety tests using fibroblasts and animals.

Introduction

Polysaccharide existing in nature is divided into structural carbohydrate and non- structural carbohydrate. Cellulose, xyloglucan, pectin, etc belong to structural carbohydrate and sucrose, starch and Fructan belong to non-structural carbohydrate. The most well-known non-structural carbohydrate is starch that consists of amylose with α-1,4 linkages of glucose or amylopectin with α-1,6 linkages of glucose. Fructan founded in natural plants or produced by microorganisms is one of the non-structural carbohydrate with β-2,6 linkages of fructose and branched chains of β-2,1 bonds based on its origin [1](Scheme 1).

Fructan existing in nature has the linked structure of one molecular of glucose and dozens to tens of thousands fructose. Fructan in plants has low molecular weight with up to 200 linkages of fructose while Fructan produced by microorganisms has high molecular weight with up to hundred thousand fructose linkages. Two main types of Fructan are the inulin with β-2,1 linkages of fructose and the levan with mainly β-2,6 linkages of fructose and different branched chains of β-2,1 bonds based on its origin. The shortest linkage of the inulin would be 1-ketose and the one of the levan would be 6-ketose.



Scheme 1. Structure of Fructan(Levan)

15% of flowering plant species naturally produce Fructan. Especially, Fructan is likely to be found in the most evolved families such as liliales, poals, astrales, campanulales, palemoniaceae, ericales, dipsacales, barley, wheat, onion, etc [2,3]. At present, 1/3 of vegetable existing in the earth is thought to contain Fructan. Particularly, from the fact that most species producing Fructan grow in the area where is dry and cold, it is known that Fructan acts as an osmoprotectant against drought and a cryoprotectant against cold damage in the plants [4,5].

Up to now, a variety of microorganisms such as *Pseudomonas sp., Xanthomonas sp., Azotobacter chroococum, Streptococcus salivarius, Basillus substilis, Actinomyces, Rothis dentocariosa, Arthrobacter ureafaciens, Zymomonas mobilis* [6-14] are known to produce Fructan. Fructan produced by microorganisms is mostly the levan type that has higher molecular weight than Fructan in plants.

It is known that Fructan (levan) has various nutritional and pharmaceutical functions such as hypocholesterolemic effect[15], promotion of metallic ion absorption[16], prevention of constipation[17] and immunomodulation effect[18].

Since Fructan may be a suitable raw material for cosmetics as well as foods and pharmaceuticals with a variety of properties mentioned above, this study investigated cosmeceutical properties of Fructan produced by *Zymomonas mobilis* and the possibility of its uses in cosmetics in order to develop it as a new cosmetic ingredient.

Materials and Methods

Materials

ELISA kit for Interleukin-1α release assay was perchased from Endogen Inc.(Boston, MA, USA)

All other chemicals were reagent-grade products obtained commercially.

Preparation of Fructan(Levan) from *Zymomonas mobilis* [19,20]

Fructan was harvested by precipitation and desiccation from the culture broth containing polysaccharides obtained from the culture medium, containing *Zymomonas mobilis*, a microorganism producing Fructan, 10% Sucrose and 1~2% yeast extract as main components, cultured for 20 ~ 24 hours at 30~37℃ and pH of 5~7 by adding alcohol (alcohol ratio of 1:3) after removing the microorganisms by centrifuging or using 0.2-0.4 micron filter.

Analysis of Fructan structure

The structure of Fructan was analyzed with Varian Gemini-200 ^{13}C-NMR spectroscopy and the results were compared with the ones in existing literatures[21, 22].

Measurement of moisturizing effect

In order to measure moisturizing effect of Fructan, two methods were employed.

Measurement of water loss in a desiccator

5g of Fructan solution(5%(w/w)) was left in a desiccator containing $CaCl_2$ at 37℃, R.H. 65% and the weight change was measured as time passed. 1% hyaluronic acid solution was also tested to compare with Fructan solution.

Measurement of moisturizing effect with Corneometer

The moisturizing effect of Fructan on skin was also measured with Corneometer CM 825 (Courage+Khazaka, C+K, Germany) in comparison with same concentration of Hyaluronic acid. 10 $\mu\ell$ of each sample were applied on 4cm^2 of antecubital fossa of volunteers and the changes of moisture content were measured at regular interval. This test was conducted at room temperature (20℃) and R.H. 20%.

Measurement of cytotoxicity

Human fibroblast and keratinocyte were used to test cytotoxicity of Fructan. Each cell line was inoculated on the 96 well plate sufficed for 100$\mu\ell$ of DMEM(Dulbecco's Modified Eagle Medium) containing 10% FBS(Fetal Bovine Serum, GIBCO BRL) with 10^4 cell/well density and incubated for 24 hours at 37℃ under 5% of CO_2. After the addition of Fructan solution, the cells were incubated again for 24 hours. Viability and proliferation of cells were measured by MTT assay. MTT solution(100$\mu\ell$) was added to each wells and incubated for 4 hours. After the overnight, 100$\mu\ell$ of 0.01M HCl containing 10% of SDS was added. The amount of formazan in the culture medium was determined by measuring absorbances at 570nm by ELISA reader.

Cell proliferation test with 3-dimensional artificial skin

Modified MTT assay based on monolayer culture system[23] was used to test cell proliferation effect in 3-D artificial skin. Culture medium was changed with new medium and 10$\mu\ell$ of SLS(Sodium Lauryl Sulfate) was added into each insert. After 4 hours, each concentration of Fructan(10$\mu\ell$) was applied and cultured for 24 hours at 37℃ under 5% of CO_2. After the washing with PBS, MTT solution(0.33mg/ml) was added into each well. After standing the well plate at an incubator for 4 hours, formazan was extracted with isopropanol at room temperature for 24 hours and the absorbance of above mixture was measured at 570nm.

Anti-inflammation test with 3-D artificial skin

Interleukin-1α release assay was carried out by measuring absorbance with ELISA kit(from Endogen Inc. Boston, MA, USA) at 450nm.

Measurement of stability of Fructan in ethanol

Fructan solution containing 5% of solid was mixed with ethanol of final concentration 10% to 50% and observed precipitation at room temperature, cold storage and 40℃ for 45 days.

Measurement of particle size.

Particle size was measured with particle size analyzer, ELS-8000(Otsuka Electronics)

Safety test on animals.

Safety of Fructan was estimated according to *CTFA Safety Testing Guideline*[24]

Results and Discussion

Analysis of Fructan structure

Chemical shift value and pattern of spectrum measured with ^{13}C-NMR are shown almost same results with known value in literature as C-1(60.2ppm), C-2(104.3ppm), C-3(76.5ppm), C-4(75.4ppm), C-5(80.2ppm), C-6(63.5ppm) and confirmed the structure of β-2, 6 combination with branch of β-2, 1[24, 25]

Measurement of cytotoxicity

Figure 1 and 2 shows the results of cell toxicity and cell proliferation effect by MTT assay, respectively. Fructan showed non toxicity against human fibroblast and cell proliferation effect for keratinocyte, suggesting that Fructan is a safe ingredient for cosmetic applications.

(Fig. 1 and 2)

Moisturizing effect

Figure 3 shows the changes of water loss of 5% Fruction solution compared with 1% hyaluronic acid measured in a desiccator. Figure 4 also shows a results of the moisturizing effects of 0.2% Fructan and 0.2% hyaluronic acid measured with Corneometer CM 825. Fructan exhibited almost same results with hyaluronic acid in comparison

(Fig. 3 and 4)

Cell proliferation test with 3-dimensional artificial skin

After the induction of skin irritation with 0.05% of SLS(Sodium Lauryl Sulfate) in 3-D artificial skin, 0.01mg/ml and 0.05mg/ml of Fructan solutions were applied and compared the proliferation effect with SLS only(Fig. 5). With above results, treated artificial skin with Fructan has a cell proliferation effect.

(Fig.5)

Anti-inflammation test with 3-dimensional artificial skin

It is known that Interleukin-1α(IL-1α) acts as a proinflammatory mediator in intercellular signal transport and induces proliferation of some cells such as osteoblast, monocyte, macrophage, keratinocyte, hepatocyte and fibroblast, by stimulations such as inflammation or infection.[25]

It is also known that IL-1α stimulates increase of arachidonic acid lipoxygenase metabolites such as leukotriene B4, 5-, 12-, and 15-HETE as well as acts as a potential inducer for reepithelialization of wound.[26] Therefore, to measure secreting quantity of IL-1α in culture medium is one of methods for evaluating anti-inflammatory effect of Fructan.

After the induction of primary skin irritation with 0.05% of SLS(Sodium Lauryl Sulfate) in 3-D artificial skin, 0.01mg/ml and 0.05mg/ml of Fructan solutions were applied and then measured the secreting quantity of interleukin-1α. Treated artificial skin with 0.01mg/ml and 0.05mg/ml of Fructan showed the decrement in quantity of IL-1α than untreated artificial skin with Fructan(Fig. 6).

As a above result, it is suggested that Fructan has a emollient effect for the skin irritation by skin irritation materials.

(Fig. 6)

Stability of Fructan in ethanol

Miscibility and stability of Fructan solution(5%of solid) mixed with a final concentration of 10 to 50%(w/w) of ethanol were measured. As a result of measurement, miscibility for ethanol was excellent and stability for each concentration of ethanol was also stable at room temperature, cold storage and 40℃ for 45 days.(Table I)

(Table I)

Measurement of particle size

Fructan partially forms nanoparticles in water. Fig. 7 shows size distribution of Fructan in water and 20% aqueous ethanol. The average particle size was measured to be 224.3 and 251.8nm, respectively.

(Fig 7)

Conclusions

In this study, we investigated some cosmeceutical properties of Fructan such as moisturizing effect, cell proliferation effect, anti-inflammation effect and cell cytotoxicity.

Fructan exhibited almost same moisturizing effect as hyaluronic acid and cell proliferation effect on human fibroblast and keratinocyte as well. Moreover, on cell proliferation test on bio-artificial skin constructed by 3-dimensional(3-D) culture after inducing primary skin inflammation with 0.5% sodium lauryl sulfate (SLS), the 3-D artificial skin treated with Fructan exhibited higher cell proliferation than the 3-D artificial skin treated with SLS only. On anti-inflammation test, the quantity of IL-1α on the 3-D artificial skin treated with Fructan was less than the one on the 3-D artificial skin treated with SLS only. As a result of these studies, Fructan has anti-inflammation effect against inflammatory reaction by a skin irritant as well as cell proliferation effect in bio-artificial skin. Fructan was also evaluated as a safe material without any toxicity in safety tests using fibroblasts and animals.

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Table Ⅰ. Stability of Fructan in ethanol

Hour / Ethanol content	7day	15day	30day	45day
10%	Stable	Stable	Stable	Stable
20%	Stable	Stable	Stable	Stable
30%	Stable	Stable	Stable	Stable
40%	Stable	Stable	Stable	Stable
50%	Stable	Stable	Stable	Stable

Figure captions

Fig. 1. Cell cytotoxicity of Fructan against human fibroblast cell

Fig. 2. Cell proliferation effect of Fructan against human keratinocyte

Fig. 3. Water loss profile of Fructan and hyaluronic acid(HA) as time passed in a Desiccator,

Fig. 4. Moisturizing effect of Fructan and hyaluronic acid(HA) measured by Corneometer CM 825

Fig. 5. Cell proliferation effect of Fructan in 3-D artificial skin.

Fig. 6. Anti-inflammation effect of Fructan in 3-D artificial skin

Fig. 7. Particles size distribution of Fructan : (a) 5% solution of Fructan in water, (b) 5% solution of Fructan in 20% of aqueous ethanol

Fig. 1

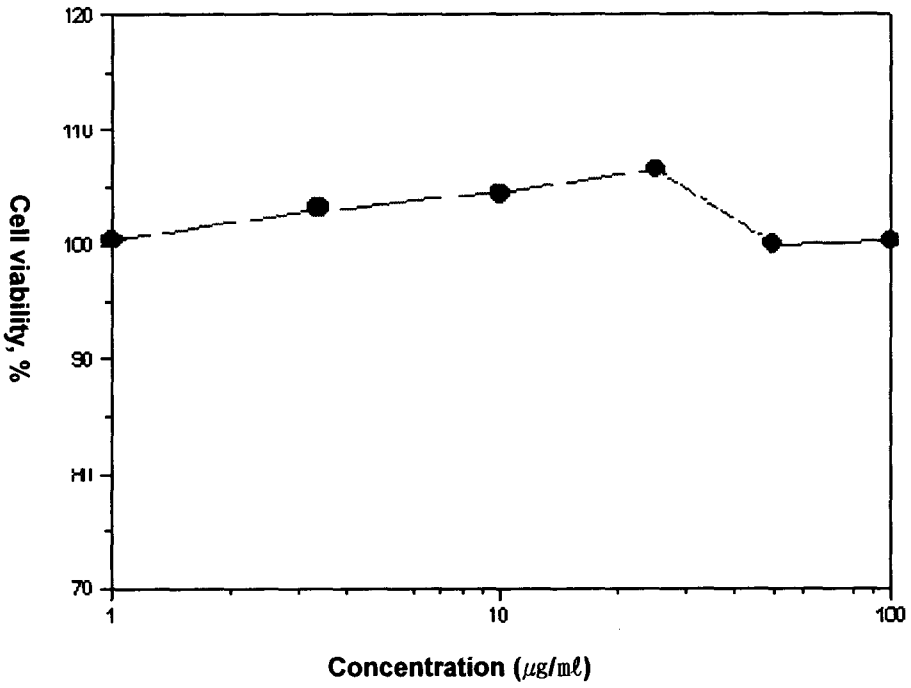

120
110
100
90
80
70
1
10
100
Cell viability, %
Concentration (μg/mℓ)

Fig. 2

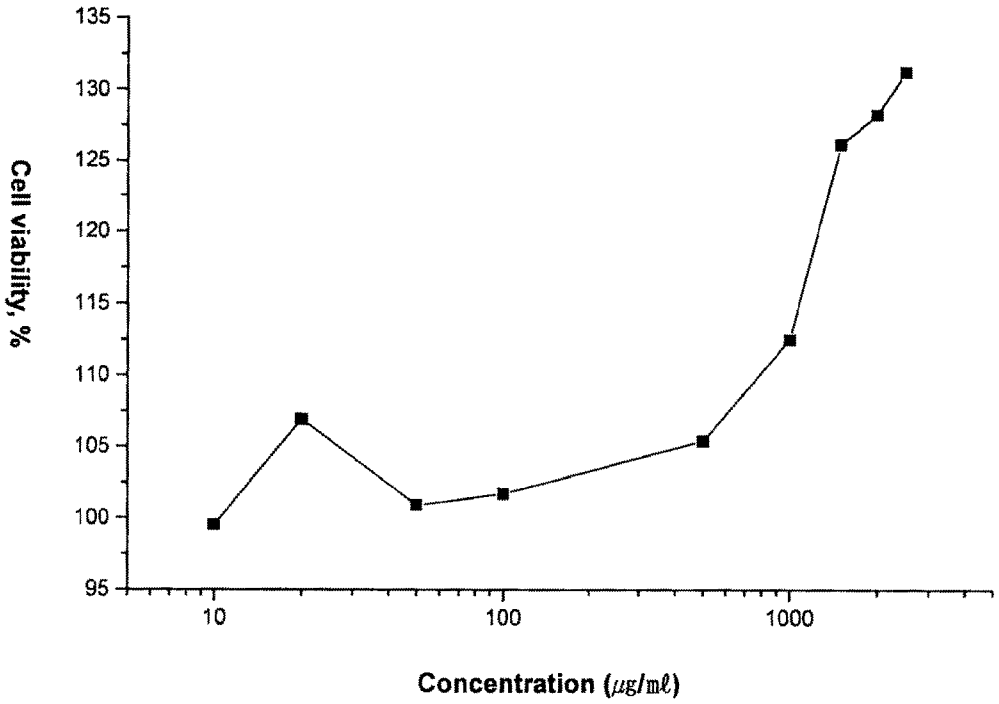

135
130
125
120
115
110
105
100
95
Cell viability, %
10
100
1000
Concentration (μg/mℓ)

Fig. 3

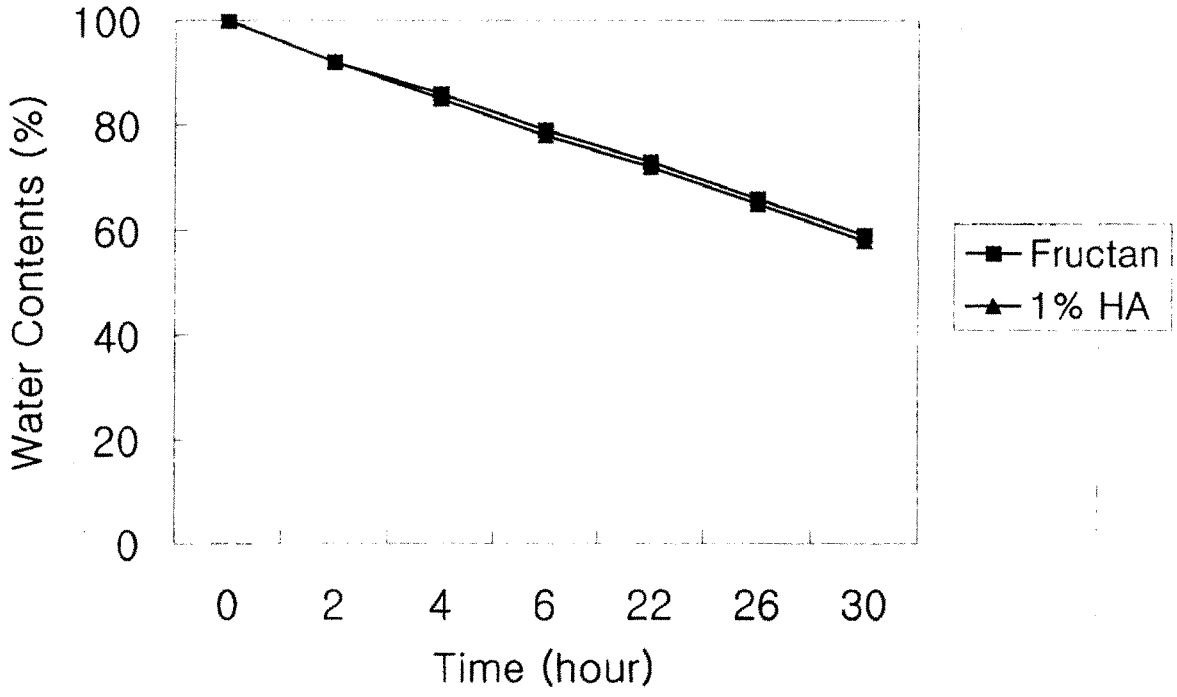

Water Contents (%)
100
80
60
40
20
0
0
2
4
6
22
26
30
Time (hour)
Fructan
1% HA

Fig. 4

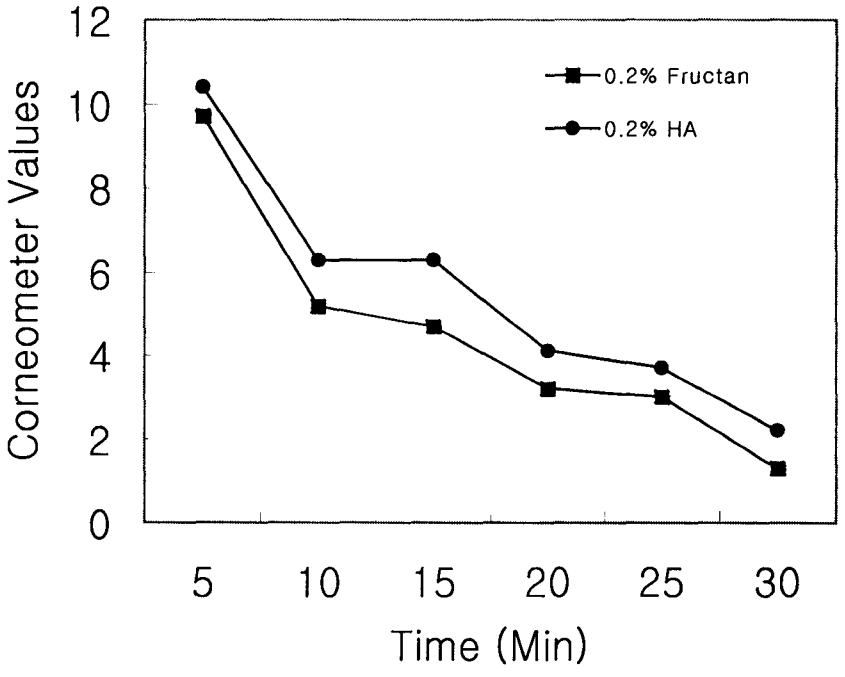

Corneometer Values
12
10
8
6
4
2
0
0.2% Fructan
0.2% HA
5
10
15
20
25
30
Time (Min)

Fig. 5

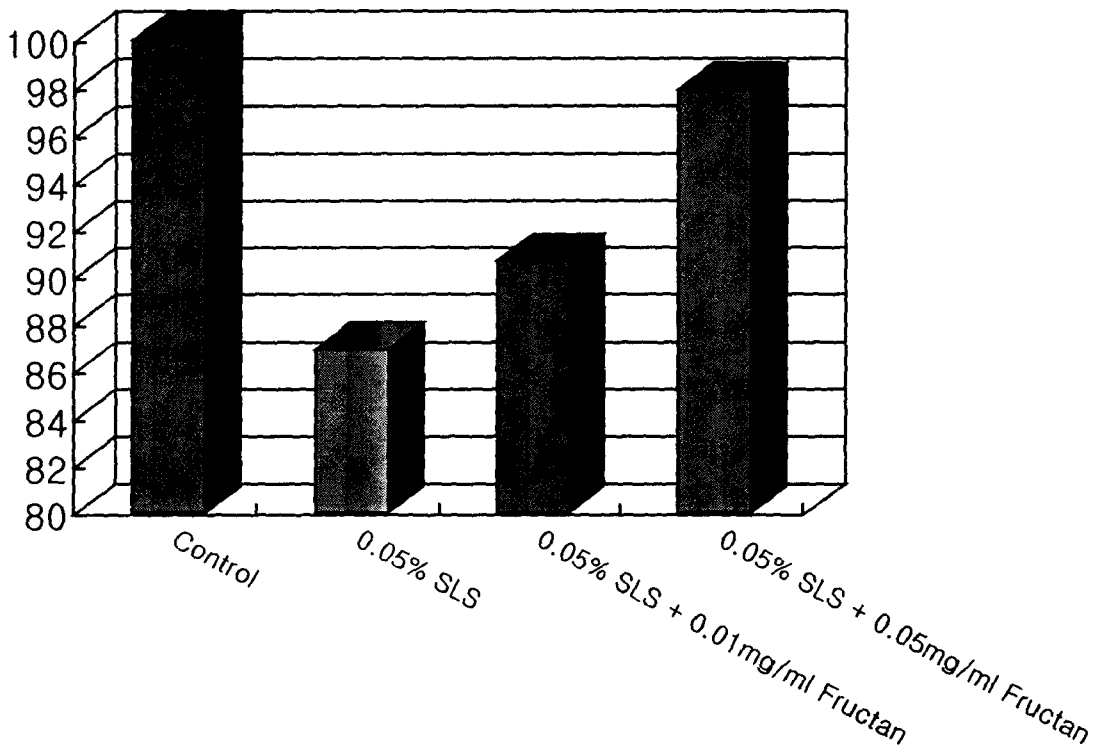

100
98
96
94
92
90
88
86
84
82
80
Control
0.05% SLS
0.05% SLS + 0.01mg/ml Fructan
0.05% SLS + 0.05mg/ml Fructan

Fig. 6

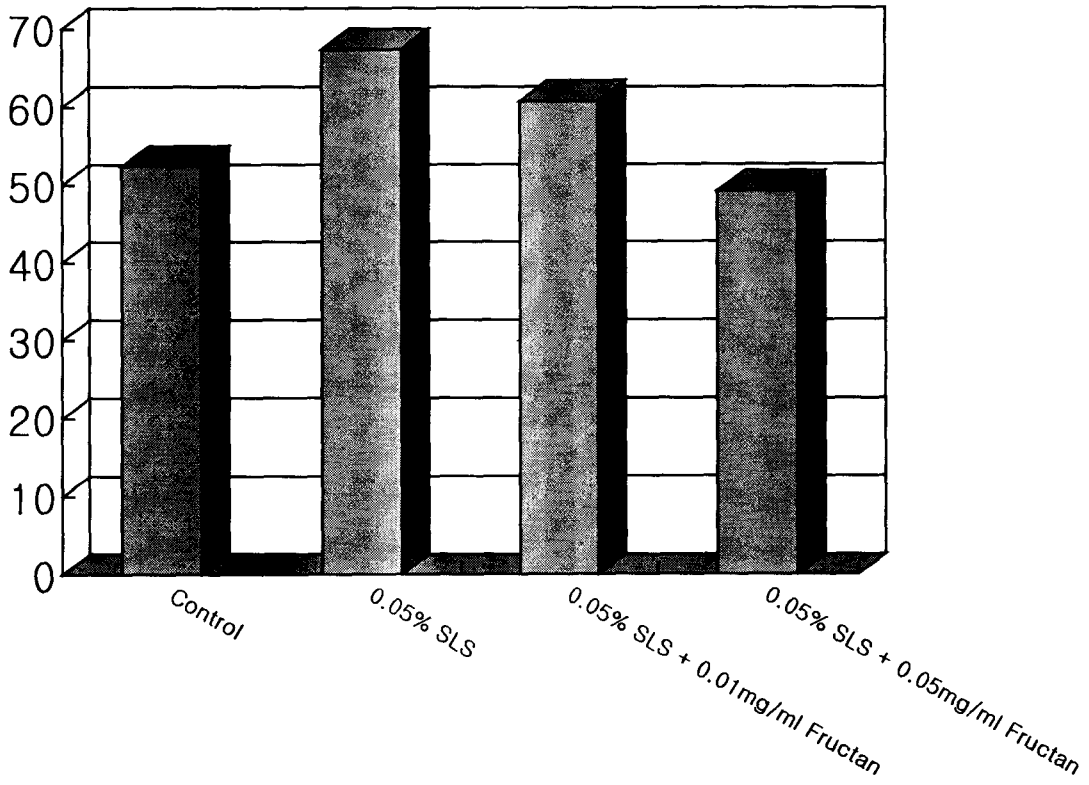
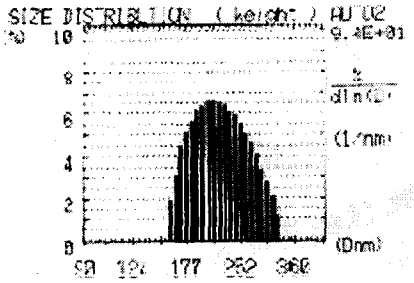
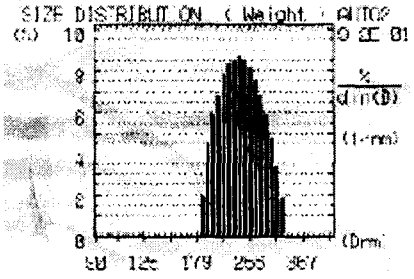

70
60
50
40
30
20
10
0
Control
0.05% SLS
0.05% SLS + 0.01mg/ml Fructan
0.05% SLS + 0.05mg/ml Fructan

Fig. 7



(a)



(b)