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인삼 사포닌 분획이 세포벽에 미치는 영향*
E. coli K-12의 세포막에 관하여

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A Study on The Effect of Ginseng Saponin Fraction on Cell Wall

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Abstract

In this experiment, observations were made on the effects of ginseng saponin, one of the major components of Korean ginseng (*Panax ginseng*, C. A. Meyer) root, on the membranes of microorganism (*E. coli* K-12), the concentration of intracelluar and extracellular cyclc AMP therein, and uptake of U-^{14}C-glucose.

When the *E. coli* were grown on media containing 0.1% ginseng saponin, the growth was faster than that of the control by about 30 minutes. The lysis of *E. coli* grown on the ginseng saponin medium increased to about double that of the control in the stationary phase. And the amount of protein and lipopolysaccharides in the outer cell membranes increased 25% and 80% respectively in comparison with the control. By electron microscope observation, it was shown that the periplasmic region of the *E. coli* grown on the ginseng saponin medium was widened. it was observed that the cellular cyclic AMP content of the *E. coli* increased significantly to the highest levels between the late exponential phase and early stationary phase. The total cyclic AMP content of *E. coli* grown on the ginseng saponin medium decreased about 50 % when compared to that of the control.

Introduction

The cell envelope of Gram-negative bacteria is made of two distinct membranes, the inner cytoplasmic or plasma membrane and the outer membrane, both of which demonstrate the usual double-tract or bilayer appearance. A peptidoglycan layer is located between the two membranes and this area between the cytoplasmic and outer membrane is referred to as the periplasmic region. Both the cytoplasmic and outer membrane are 75 A° thick and peptidoglycan layer is about 25-30Å. The outer membrane, the most outer layer of Gram-negative bacteria, consists of proteins, lipopolysaccharide (LPS) and phospholipids.[1] The outer membrane fraction contains approximately 60% of the protein of the total membrane, 50% of the phospholipid and 90% of the lipopolysaccharide.[2] Outer membrane proteins by definition were originally characterized after the separation of bacterial membranes by sucrose-density-gradient centrifugation.[3,4]

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There are many studies involving both higher animals and microorganism on cyclic AMP. Makman and Sutherland[5] first investigated the effects of C-AMP which is required for the synthesis of a numbur of inducible enzymes that catalyze catabolic reaction in *E. coli* as well as for the formation of flagella.[5,6,7] The mechanism by which the cellular concentration of C-AMP is regulated is not understood at present. Evidently the activities of at least two enzymes, i. e., the synthetic adenyl cyclase and the degradative C-AMP phosphodiesterase, affect the cellular concentration of the cyclic nacleotide. It has been suggested that the release of C-AMP into the medium may also play a regulatory role.[6] Buettner *et al*[8] suggested that the C-AMP phosphodiesterase can not be the primary site of regulation and also suggested that catabolite repression is regulated by rather suprisingly by the threshold concentrations of C-AMP; e, g., an increase of the cellular concentration of C-AMP from 1.2×10^{-5} M to 2.3×10^{-5} M permitted escape from catobolite repression. Now there are deep concern at the regulation of the cellular concentration of C-AMP and mutants being used for determination.[9,10]

In this paper we describe experiments on the effect of ginseng saponin on cell growth, cell envelope, intracellular and extracellular C-AMP, and radioactivity of TCA extractable fraction.

Materials and Methods

Preparation of saponin:

20.15g of ginseng saponin were obtained from 1kg of powdered Korean white ginseng roots (Keumsan, 5 years, 50 pieces/300g) using the modified procedure described elsewhere.[11]

Bacterial strain and medium:

EC-1-lyophilized cells of strain K-12 *E. coli* (sigma) alkaline phosphatase. The bacteria were maintained as lyophilized stock and grown in batch cultures in minimal medium with 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02%$MgSO_4$, and 0.36% glucose or 0.36% lactose. Condition of growth: The media were sterilized by autoclaving. The glucose or lactose was sterilized seperately and added aseptically. When ginseng was added to a liquid culture, the ginseng saponin solution was passed through a sterilized membrane filter (0.45μm pore size; 25mm diameter). Bacteria were grown aerobically at 37℃ in a shaking incubator and observation of cell growth used by coleman Junior Ⅱ spectrophotometer at 420nm.

Lysis of *E. coli* cell walls:

A Bausch and Lomb spectronic 20 was used at 450nm. Dry cell, for substrate, suspended on 0.1M phosphate buffer (pH7.0). Dry lysozyme-HCl, for enzyme, suspended on same buffer with total reaction volume of 3mℓ. Substrate concentration was 0.2mg/mℓ and concentration of enzyme was 0.4mg/mℓ.

Isolation of the outer membrane:

The procedure was based on equilibrium density gradient centrifugation of the total membrane faction obtained by lysis of the lysozyme-EDTA spheroplast. The outer membrane was prepared using a slight modification of the method of Osborn *et al.*[12] Cells were harvested by centrifugation at 10,000rpm for 5min. at 0-4℃. After rapid washings with distilled water, they were resuspended in about ten volumes of cold 0.75M sucrose-10mM Tris-HCl buffer, pH7.8. Then lysozyme was immediately added to give a final concentration of 0.4mg/mℓ (0.2mℓ of 2mg/mℓ

solution /ml of cell suspension), and the mixture incubated at 25℃ for 2min. The suspension was then slowly diluted with 2 volumes of cold 2.5mM EDTA, pH7.5. The lysates were centrifuged cold for 20 min. at 1,200×g to remove intact cells, and supernatant fraction then centrifuged for 20min at 20,000×g and the membrane pellets were carefully suspended in the same buffer to a final volume of approximately 1/3 that of the original lysate. The membranes were collected by 5%−60% sucrose gradient centrifugation for 1 hour at 38,000×g at 4℃. The outer membrane fractionated between the 5% and the 60% sucrose. This fraction was washed three times with original buffer solution and used for the following experiment.

The protein and lipopolysaccharide content of outer membrane:

The collected membrane fraction was lyophilized until the weight remained constant. The protein content was determined by the method of Lowry *et al* [13], employing bovine serum albumin (F5) as the standard. The lipopolysaccharide content was estimated by determination of KDO in the membrane. For estimation of KDO, the membrane fractions were precipitated with cold 10% trichloroacetic acid(5mℓ), collected by centrifugation at 4℃ for 10 min at 20,000×g and washed twice with 5mℓ of H_2O by centrifugation. The precipitate was suspended in 0.7mℓ of 0.018N H_2SO_4 and hydrolyzed at 100℃ for 2min. to liberate the KDO from the lipopolysaccharide. KDO was determined directly on the hydrosate by the thiobarbituric acid method.[14]

Electron microscopy:

The cell surface was examined under a Hitachi HS-73 electron microscope. Samples for thin sectioning were prefixed with 2% glutaraldehyde in 0.1M sodium phosphate butter (pH7.2) for 2 hrs. They were then washed with same buffer several times. Postfixation was made with 1% O_sO_4 in the same buffer at 0℃. The samples were dehydrated using an ascending series of ethanol and embedded in Epon. Sections cut on a Sovall MT 2-8 ultramicrotome were first stained with 2% uranyl acetate solution overnight and subsequently stained with lead citrate solution for 5min.

Measurement of C-AMP:

The sample for the measurement of C-AMP was carefully obtained by the method of Bernlohr, Haddox and Goldberg.[9] For the determination of intracellular concentration of C-AMP, samples equivalent to 2.5 to 5.0mg of protein were removed from the culture at the indicated time and filter, under vacuum, on Millipore filters (0.45μm pore size;25mm diameter). After washing the filtered cells with 5mℓ of the growth medium (minus the carbon source), the filters were placed in dishes containing 5% trichloroacetic acid. After cooling at 4℃ for at least 30 min, cell debris were then removed from the extracts by centrifugation at 30,000×g for 10 min. Trichloroacetic acid was extracted 5times from the clear supernatant solutions using equal volumes of ether, and the aqeous phase was evaporated to dryness in vacuo. These are suspended in 0.05M Tris/EDTA buffer containing 4mM EDTA, pH7.5.

Samples(50μℓ) of the extracts, diluted in the same buffer, were then assayed in duplicate for their concentration of C-AMP. For the determination of the extracellular C-AMP, a fraction of the filtrate was treated in the same manner. The measurement of C-AMP was made with the cyclic AMP Assay Kit 432(Amersham). This method is based on the competition between unlabelled C-AMP and a fixed quantity of tritium labeled C-AMP for binding to a pro-

tein which has a high specificity and affinity for C-AMP. A standard curve was obtained by the assay of solution prepared by serial dilutions of the standard solutions. The standard C-AMP solution gave a range of 1 pmole to 16 pmoles. The radioactivity of the protein-bound ^{3}H-C-AMP on the filters was determined by liquid scintillation counter (Packard Liquid Scintillation Counter) after placing the filter in the counting vial. Presentation of the intracellular and extracellular concentrations of C-AMP in units of molarity is based on the method of Buettner, Eva Spitz and Richenberg.[8]

Measurement of radio activity of TCA extractable fraction:

20mℓ of u-^{14}c-glucose solution (20μci) was added to the 180mℓ of glucose medium. For the determination of intracellular radio activity, the incubated cultures were harvested by the Millipore filters from the exponential phase to the stationary phase. The filters were placed in dishes containing 2mℓ of 5% trichloroacetic acid solution and after cooling at 4℃ for 30min., the solutions were centrifuged at 30,000×g for 10min. 800μℓ of the supernatent was collected with micropipette and radioactivity was determined by scintillation counting.

Results

Cell lysis:

When the *E. coli* were grown on the media containing $1\times10^{-4}\%$ – $1\times10^{-1}\%$ ginseng saponin, the growth of *E. coli* increased in proportion to the increase in concentration of the ginseng saponin. The exponential growth on the medium containing $1\times10^{-1}\%$ ginseng saponin was faster than that of the control by about 30 minutes (fig. 1). Assuming that this effect was related to the bacterial cell membrane, we compared cell lysis for cell grown on the medium containing ginseng saponin and on the control medium (Table 1). The data indicate that the lysis of *E. coli* cells grown on the ginseng saponin medium with lysozyme increased about 2 times compared to that of the control.

Outer membrane component change:

The amounts of protein and lipopolysaccharide in the outer membrane increased 25% and 80% respectively in comparison with the control (Table 2). By electron microscopic observation, it was noted that for the cell grown on glucose or lactose medium containing the saponin, the periplasmic region widened.

Concentration of C-AMP in bacteria and medium:

Our results, like those of Makman and sutherland's[5] experiment, indicate that the cellular concentration of C-AMP rises sharply as the source of carbon disappears from the medium. In this experiment, *E. coli* K-12 was grown on a limiting concentration of glucose or lactose and ginseng saponin solution was added to this control medium as the test medium. The intracellar and extracellular concentrations of C-AMP were monitored at 2hr intervals. The intracellular concentration of C-AMP in *E. coli* grown on glucose medium is double that of the test containing ginseng saponin solution during the stationary phase, whereas in the case of extracellular concentration of C-AMP secreted into the medium, this is the same as that of the test medium (Fig. 3). On the other hand, the intracellular concentration of C-AMP in *E. coli* grown on a lactose medium did not change for the test. But the extracellular concentration of

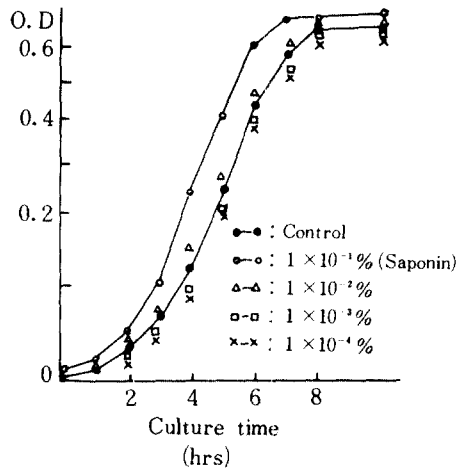

Fig. 1 Growth curve of *E. coli* K-12 in glucose medium.

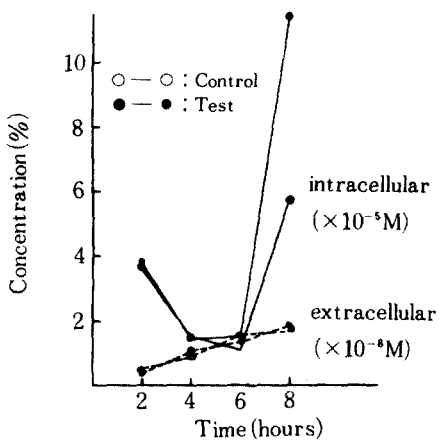

Fig. 3 The intracellular & extracellular concentration of C-AMP in glucose medium.

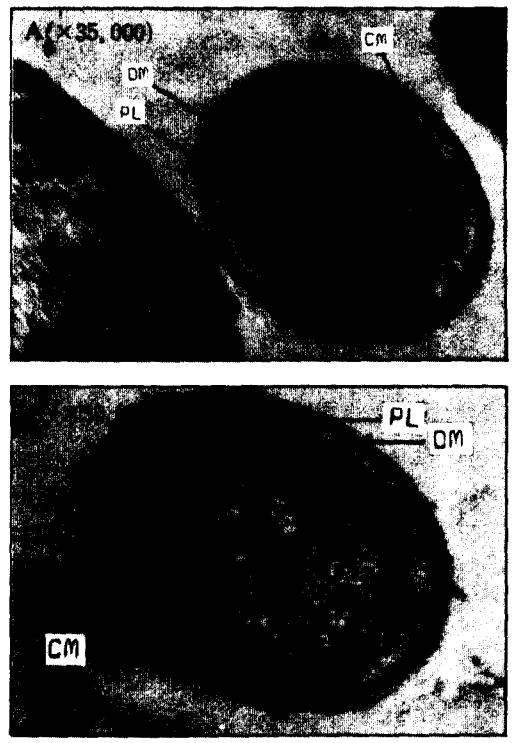

Fig. 2 Electron micrographs of thin section of *E. coli* K-12 grown with glucose medium (A) and lactose medium (B) containing ginseng saponin*

OM : Outer membrane
PL : Periplasmic region.
CM : Cytoplasmic membrane
* Final concentration of ginseng saponin was 0.1%

Table 1. Lysis of *E. coli* grown with/without ginseng saponin* in glucose medium by lysozyme.

Medium \ Time (min)	Optical change/min 0 – 1	1 – 2	2 – 3	Relative lysis activity
Glucose (exp)**	0.024	0.008	0.011	100
Glucose + saponin (exp**)	0.026	0.014	0.005	105
Glucose (sta***)	0.018	0.004	0.002	100
Glucose + saponin (sta***)	0.038	0.010	0.006	225

* Final concentration of ginseng saponin was 0.1%.
** Early exponential phase
*** Early stationary phase

Table 2. The contents of outermembrane component of *E. coli* K-12 grown with/without ginseng saponin in glucose medium at 37℃ for 6 hours.

Protein (μg protein perg weight of dry cell membrane)

medium	Protein	Relative amount
Control	380.0	100
Saponin*	450.0	125

Lipopolysaccharides

medium	O.D value	Relative amount
Control	0.160	100
Saponin*	0.288	180

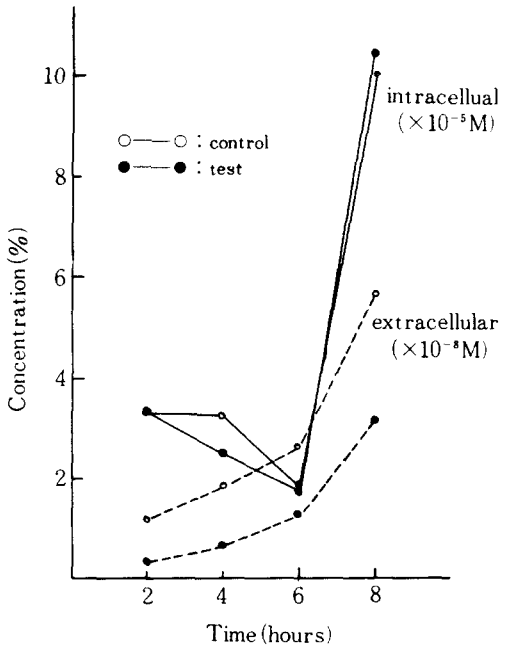

Fig. 4 The intracellular & extracellular concentration of C-AMP in lactose medium

C-AMP released into medium is double that of the test during all phases (Fig. 4). In each medium, both glucose and lactose, the control has more C-AMP than that of the test.

TCA extractable radioactive fraction:

The intracellular radioactivities of the test were higher than that of the control from 2hr to 6 hr, especially at early stage (Table 3).

Table 3. Radioactivities of TCA extractable fraction in *E. coli* K-12 grown on mixture of glucose and U—^{14}C—glucose with/without ginseng saponin*

Medium / Time (hr)	Control (cpm)	Saponin (cpm)
2	168	447
3	192	270
4	148	219
5	198	262
6	292	291

* Final concentration of ginseng saponin was 0.1%

Discussion

As the ginseng saponin affects cell growth, we observed changes in the cell membrane. We think that the ginseng saponin has a function similar to EDTA because cell lysis by lysozyme was increased when ginseng saponin was added to the control medium. This fact suggested that ginseng saponin affected the cell membrane, therefore we studied the outer membrane components. In this expriment, we observed that both the LPS and the outer membrane protein content of *E. coli* K-12 increased when the ginseng saponin was present. This indicated that the ginseng saponin affects the lysozyme function indirectly by changing the cell membrane component. As the observations reported were in general agreement with our earlier findings, we observed the membrane structure by electron microscrope. This showed that the periplasmic region, which is located between the cytoplasmic and outer membranes, widened significantly. It is well known that bacterial lysis by lysozyme action is a result of the hydrolytic cleavage of the N-acetylmuramyl-N-acetylglucosamine linkage in glycan strands which destroys the integrity of the peptidoglycan fabric. We suggest that the periplasmic region was widened by the ginseng saponin making it easier for lysozyme to react with.

Pastan and Perlman[15] found that exogenously added C-AMP overcome catabolite repression in *E. coli*. This finding suggested the possible existence of an inverse relationship between the cellular concentration of C-AMP and the intensity of the catabolite repression. It became of interest, therefore, to determine the intracellular and extracellular concentrations of C-AMP under a variety of conditions of growth. It has been previously reported that cell grown on lactose had more C-AMP than grown on glucose. Our experiment, as well as Makman's[5] experiment, showed that the cellular concentration of C-AMP rose about 7 times as the source of carbon disappeared from medium. In each medium, in the case of the cells grown on glucose, the intracellular concentrotion of C-AMP decreased to half while the extracellular C-AMP amount did not change when ginseng saponin was added. Whereas the intracellular concentration of C-AMP for cells grown on lactose did not change, the extracellular C-AMP decreased to half by addition of ginseng saponin. As our results indicate that a decrease of the intracellular concentration of C-AMP was not found immediately in the medium, it is more likely that the ginseng saponin dose not activate the release of C-AMP into the medium which is one of the regulation mechanisms of C-AMP considering results (Fig. 3). And previous experiments suggests that the C-AMP phosphodiesterase can not be the primary site of regulation[8]. One may assume, therefore, that the ginseng saponin inhibits the activity of adenylat cyclase.

Inconclusive as the results are, it is likely that the higher radioactivity of the test relates to rapid uptake of glucose or TCA extractable compounds are more produced than those of the control.

Further experiments on relationship among growth, cell lysis, electronmicroscope observation, C-AMP concentration and TCA extractable fraction are desirable to clarify the physiological role of the ginseng saponin and these results mayhelp the study on animal cell membrane.

요 약

E. coli K-12에 대한 인삼 saponin의 영향. 본 연구에서는 한국산 인삼 (*Panax ginseng* C. A. Meyer)의 유효성분이라고 보는 인삼 saponin을 가지고 미생물의 세포막에 대한 작용과 세포내, 세포외의 C-AMP함량, U-^{14}C-qlucose uptake amount에 미치는 영향을 살펴본 결과 다음의 결론을 얻었다.

1. 최종 농도 0.1%의 인삼 saponin을 첨가한 배지에서 자란 세포의 경우 대조군에 비해 증식이 약30분 촉진되었다.
2. 인삼 saponin이 첨가된 배지에서 배양된 세포의 경우 stationary phase에서 약 2배의 lysis activity의 증가를 가져왔다.
3. 인삼 saponin이 첨가된 배지에서 자란 cell의 outercell membranes에서 protein의 양과 lipopoly saccharides의 양은 각각 25%와 80% 증가했다.
4. 인삼 saponin이 첨가된 배지에서 자란 세포에 대한 전자현미경의 관찰에 있어서 glucose배지에서 자란것이나 lactose배지에서 자란것, 둘 다에서 대조군에 비해 periplasmic region이 상당히 넓다는것을 알수 있다.
5. 시간에 따라 세포내의 C-AMP의 양을 조사해본 결과 carbon source가 고갈될 쯤인 exponential phase에서 stationary phase로 도달하는 순간에 약 7배의 C-AMP 농도의 증가를 가져왔다. 또한 인삼 saponin을 첨가한 배지에서 자란 세포의 총 C-AMP의 양은 대조군의 50% 정도로 현저히 감소하였다.
6. 인삼 사포닌에서 배양한 시험군의 TCA extractable fraction은 대조군보다 방사성 양이 높은 경향을 보였다.

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