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Effect of Ginseng Saponin Fraction on Cleavage of pBR322 by Several Restriction Endonuclease

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인삼 Saponin 분획이 제한효소에 의한 pBR322 절단에 미치는 영향

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Abstract

Attempts were made to see if we could cut more pBR322 in the presence of ginseng saponin fraction in connection with possibly for shortening the enzymatic reaction time and the amount of the enzyme to be used. The following results were obtained restriction endonucleases such as AccI, XhoII, SaII, and HincII were observed to cut pBR322 efficiently at 10^{-1}% ginseng saponin fraction. In case of BamHI, 10^{-2}% ginseng saponin fraction was observed to the most effective concentration. Such cumulative results suggest that ginseng saponin fraction would play important role as far as the cleavage of pBR322 for short period by endonucleases is concerned.

Introduction

Saponin fraction obtained from root of *Panax ginseng* is known to be the second large one to polysaccharides as far as the amount of component is concerned. The fractions also were known to elevate the activity of enzymes such as glutathione reductase and peroxidase[1] dehydrogenase[2~5] , and DNA-dependent RNA polymerase[6] *in vitro* and *vivo*. In any case described above, a apropriate concentration is required to elevate the enzymatic activity. At above and below the concentration of the saponin, the effect of the saponin is known to be inhibitory and innocuous, respectively.

During the last decade the newly developed techniques of genetic manipulation have enable us to isolate a single gene. As result of the isolation of genes such as those for globin, insulin and the immunoglobins a series of fundamental advances have been made

in our understanding of the organization of the eukaryotic genome. The foundation for these advances were laid in the early 1970s by the discovery of enzymes (restriction endonucleaes) which could cut DNA at specific sites, other enzymes (ligases) which allowed DNA molecules from different sources to be joined, and reverse transcriptase which synthesized complementary DNA using a appropriate mRNA. Also crucial was the development, at about the same time, of a way of introducing exogenous DNA into vector (plasmid). By combining these techniques, it was possible to insert a foreign DNA fragment into vector and then to amplify the hybrid molecule by propagating it.

The present paper deals with the effect of saponin fraction on the activity of several restriction endonucleases vital for genetic engineering in connection with saving amount of enzymes to be used and reducing the enzymatic reaction time.

Materials and Methods

Materials

Ethidium bromide, cesium chloride, Agarose (Typ II), Tris, 2-mercaptoethanol, bovine serum albumin, phosphocellulose, Heparin agarose, DEAE-Sephacel, and Bromophenol Blue were purchused from the Sigma Chemical Co. Hydroxylapatite was purchased from the Bio-Rad. Preparation of ginseng saponin was carried out by a previous method[7]. Other chemicals were of reagent grade.

Isolation of pBR322

25 ml of the late log culture was inoculated into 500 ml of LB medium prewarmed to 37°C with the appropriate antibiotic in a 2-liter flask. Cultures were grown for 2.5 hr at 37°C with vigorous shaking until the culture reaches and OD600-0.4. 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) were added to the culture. The final concentration of chloramphenicol in the culture was 170 ug/ml. Cultures were grown at 37°C with vigorous shaking for a further 12-16 hr. The bacterial cells were harvested by centrifugation at 4000 g for 20 min at 4°C. Following method was done using a modification of the method used by previous workers[8]. The bacterial pellet was suspended in 10 ml of an ice-cold solution of 10% sucrose in 50 mM Tris-HCl (pH 8.0) and 8 ml of 0.25 M EDTA was added to the suspension. The suspension was mixed by inverting the tube several times and then placed on ice for 10 min. 4 ml of 10% SDS are added to the suspension. Then 0.6 ml of 5 M NaCl was added and mixed gently but thorougly. The suspension was placed on ice for at least 1 hr. The high-molecular-weight DNA and bacterial debris were removed by centrifugation at 1500g for 30 min. The supernatant was extracted twice with phenol/choloroform. After ex-

traction, the agueous layer was transfer to a clean tube. This solution was mixed with 2 volume of ethanol and let stand at -20°C for 1-2 hr. The nucleic acid was recovered by centrifugation at 1500 g for 20 min at 4°C. The precipitate of DNA was dissolved in a total volume of 8 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). For every milliliter, 1 g of solid cesium chloride was added to DNA solution and mixed gently until all of the salt is dissolved. Then, 0.8 ml of a solution of ethidium bromide (10 mg/ml in H_2O) was added for every 10 ml of cesium chloride solution to the solution. The cesium chloride solution was transfered to a tube suitable for ultracentrifugation at 35,000g for 36 hr at 20°C. The lower band of DNA was required. An equal volume of 2-butanol was added to DNA sample and mixed well. The upper phase was removed by centrifugation at 1,600 g for 1 min. The purified DNA was stored at -20°C.

Preparation of endonucleases

The preparation of BamHI was done using a modification of the method used by previous workers[9][10][11][12][13]. Briefly, 10 g of frozen cells were suspended in 20 ml of S buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM NaN_3). The suspension was sonicated in an ice-water bath with Lab Line Instrument, Inc. for three 20-sec bursts. Cellular debris was removed by centrifugation at 15,000g for 20 min. The supernatant was mixed with phosphocellulose resin. The mixture was then poured into a column with a final bed dimension of 2.5 x 4 cm, washed with approximately 250 ml of S buffer and eluted with a 500 ml linear gradient of 0 to 1.0 M KCl in S buffer at a flow rate of 10 ml/hr. BamHl was eluted between 0.3 and 0.5 M KCl. Active fractions were pooled, and diluted at least twofold with 1.0 mM EDTA, 1.0 mM NaN_3 and 10.0 mM 2-mercaptoethanol, and loaded directly on a Hydroxylapatite (Bio Gel HTP) column (1.2 x 8 cm) that has been equilibrated with 25 mM potassium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol, 1 mM EDTA, and 1 mM NaN_3. After washing with 100 ml of the same equilibration buffer, enzymatic activity was eluted with a 100 ml linear gradient of 25 to 250 mM potassium phosphate buffer, pH 7.0, in the equilibration buffer. BamHl was eluted between 100 and 150 mM potassium phosphate. The active fractions were pooled and dialyzed 1 mM EDTA, 200 mM NaCl and 50% glycerol and stored at -20°C.

The isolation of SalI was made using a modification of a previous method[14][15]. 10 g of frozen cells were suspended in 12 ml sonication buffer containing 2.5 mg/ml of lysozyme, 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol. The suspension was sonicated in an ice-water bath with Lab Line Instrument, Inc. for four 30-sec bursts. Cellular debris was removed by centrifugation at 100,000 g for 1 hr. The supernatant was diluted with one volume of PC buffer (10 mM potassium phosphate, pH 7.5, 10mM 2-mercaptoethanol, 0.1 mM EDTA). This solution was applied to a phosphocellulose

column (2x20 cm) equilibrated with PC buffer. After washing with approximately 200 ml of PC buffer, elution carried out with a 500 ml linear gradient of 0-0.7 M NaCl in PC buffer. Activity appeared in a peak between 0.4 and 0.5 M NaCl. The active fractions were dialyzed against PC buffer and applied to Hydroxylapatite column (2.5x6 cm). After extensive washing, elution was carried out with a 60 ml of PC buffer containing 0.2 M NaCl. The active fractions were pooled and dialyzed against 20 mM potassium phosphate buffer (pH 7.4), containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 200 mM NaCl and 50% glycerol and stored at -20°C

The preparation of XhoII is following method. 20 g of frozen cells were suspended 40 ml of buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol. The suspension was sonicated in an ice-water bath with Lab Line Instrument, Inc. for seven 20-sec bursts. The extract was then centrifuged for 1 hr at 15,000g. 10% solution of streptomycin sulfate was added dropwise to the supernatant with constant stirring for 2 hr to a final concentration of 1.0%. After centrifugation (20 min, 10,000g), the precipitate was discarded. The supernatant is dialyzed against PC buffer (10 mM potassium phosphate, pH 7.4, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 5% glycerol). The dialysate was applied to phosphocellulose column (3x20 cm) at a flow rate of 10 ml/hr previously equilibrated with PC buffer. After application of the sample, the column was washed with approximately 200 ml of PC buffer and eluted with a 400 ml linear gradient of 0 to 1.0 M KCl in PC buffer at a flow rate of 25 ml/hr. XhoII was eluted between 0.6 and 0.7 M KCl. Active fractions were pooled and dialyzed against 2 liters of PC buffer for 12 hr and applied to Heparin agarose column (2x10 cm) at a flow rate of 8 ml/hr. The column is washed with 100 ml of PC buffer and eluted with a 200 ml linear gradient of 0 to 0.8 M KCl in PC buffer at a flow rate of 20 ml/hr. Enzyme activity is at 0.35 to 0.5 M KCl. Active fractions were pooled and dialyzed against PC buffer. The dialysate was applied to DEAE-sephacel column (2x3 cm) at a flow rate of 5 ml/hr. The column was washed with 80 ml of PC buffer and eluted with a 100 ml linear gradient of 0 to 0.6 M KCl in PC buffer at a flow rate of 15 ml/hr. The peak of enzyme activity was at 0.2 to 0.3 M KCl. Active fractons were pooled and dialyzed against 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 200 ug/ml BSA, 50% glycerol and stored at -20°C.

AccI was purchased from the BRL.

HincII was purchased from the BRL.

Enzyme assay

The assay of BamHl was carried out by a previous method[9]. The enzyme assay mixtures (20 ul) contain 2 ug of pBR 322, 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 100 mM NaCl, 2 mM 2-mercaptoethanol, 0.01% bovine serum albumin, 0%-10^{-6}% of saponin fractions and 1 ul of enzyme. Incubation was done for 15 min at 37°C.

The assay of SalI was carried out by a previous method[14]. The enzyme assay mixtures (20 ul) contain 2 ug of pBR 322, 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 175 mM NaCl 0.2 mM EDTA, 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin 0%-10^{-6}% of saponin fractions and 1 ul of enzyme. Incubation was done for 10 min at 37°C.

The assay of XhoII was arried out by a previous method[16]. The enzyme assay mixtures (20 ul) contain 2 ug of pBR 322, 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin, 0%-10^{-6}% of saponin fractions and 1 ul of enzyme. Incubation was done for 40 min at 37°C.

The assay of AccI was carried out by a previous method[17]. The enzyme assay mixtures (20 ul) contain 2 ug of pBR 322, 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin, 0%-10^{-6}% of saponin fractions, and 0.5 units of enzyme. Incubation was done for 10 min at 37°C.

The assay of HincII was carried out by a previous method[18]. The enzyme assay mixtures (20 ul) contain 2 ug of pBR 322, 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol, 0-10^{-6}% of saponin fractions, and 0.5 units of enzyme. Incubation was done for 10 min at 37°C.

All reactions were stopped by addition 5 ul of dye mixture containing 40% sucrose, 0.25% Bromophenol blue, 0.1 M EDTA. Samples were analyzed for DNA cleavage by electrophoresis on a 1% agrose gel for 30 min at 120 volt.

Results and Discussion

The most commonly used plasmid vectors are pBR322 and other derivatives of pMBI, a plasmid closely related to Co1EI-K30. pBR322 consists of the essential replication of pMBI to which two drug-resistance genes, coding for ampicillin resistance and tetracycline-resistance, have been added by ligating fragments of R plasmids. More than 10 restriction enzymes including SalI, BamHI cut pBR322 at single site.[19] In our experiment, the well known plasmid, pBR322 was used as substrate for several restrictin endonucleases. The following cleaved products by reducing the incubatin time in order to see the more clear effect of ginseng saponin fraction on the enzymes.

At 10^{-1}% of ginseng saponin fraction on restriction endonuclease AccI was observed to cut more pBR322 than those of the control and at the rest of ginseng saponin concentration (Fig. 1). The cleaved pBR322 was observed to move a little from the well, and would be completely cleaved if the enzymatic reaction time was extended. Anyhow, the best concentration of ginseng saponin fraction for endonuclease AccI to cleave efficiently was 10^{-1}%. Such trends as the best concentration for enzyme were also similar to the previous papers.[2-5,7] Also same trends were observed in case of XhoII at 10^{-1}%

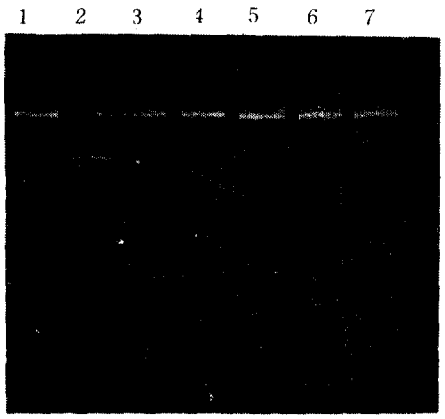

Figure 1. Agarose gel electrophoresis restriction pattern of pBR322 after the action of AccI for 10 min incubation. Lane 1 is control, lanes from 2 to 6 are products digested by AccI in the presence of the saponin fraction (10^{-1}-10^{-6}%). The amount of pBR 322 used as a substrate was 2 μg.

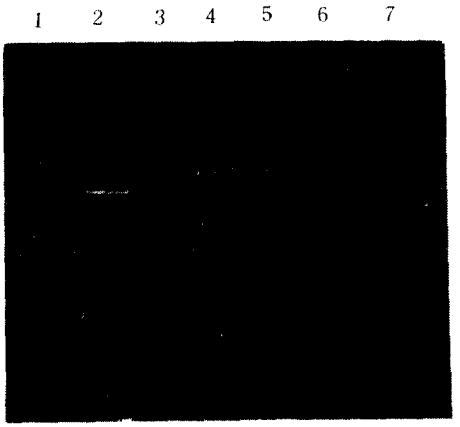

Figure 2. Agarose gel electrophoresis restriction pattern of pBR322 after the action of XhoII for 40 min incubation. Lane 1 is control, lanes from 2 to 6 are products digested by XhoII in the presence of the saponin fraction (10^{-1}-10^{-6}%). The amount of pBR 322 used as a substrate was 2 μg.

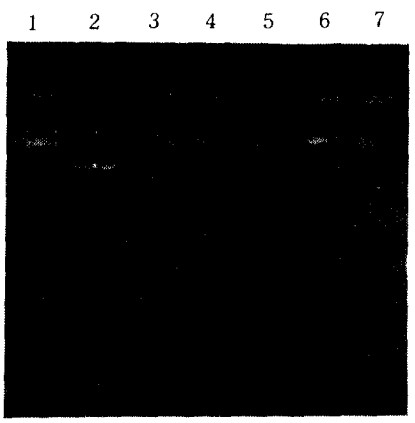

Figure 3. Agarose gel electrophoresis restriction pattern of pBR322 after the action of SalI for 10 min incubation. Lane 1 is control, lanes from 2 to 6 are products digested by SalI in the presence of the saponin fraction (10^{-1}-10^{-6}%). The amount of pBR322 used as a substrate was 2 μg.

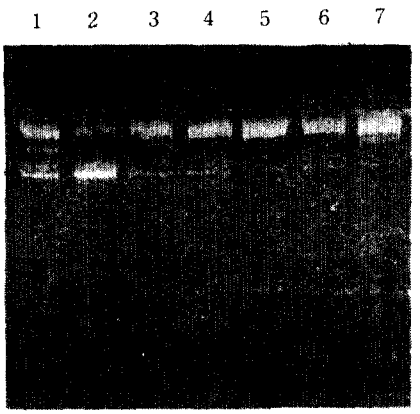

Figure 4. Agarose gel electrophoresis restriction pattern of pBR322 after the action of HincII for 10 min incubation. Lane 1 is control, lanes from 2 to 6 are products digested by HincII in the presence of the saponin fraction (10^{-2}-10^{-6}%). The amount of pBR322 used as a substrate was 2 μg.

ginseng saponin fraction (Fig. 2). The fast moving band was the product cleaved by XhoII from pBR322. More cleaved products by SalI and HincII were observed at 10^{-1}% ginseng saponin fraction than those of the control and at the rest of ginseng saponin concentration (Figs. 3 and 4). Similar results were also observed even in case of other enzyme[7]. However, BamHI was observed to cut more pBR322 at 10^{-2}% (Fig. 5) whereas the rest of endonucleases at 10^{-1}% ginseng saponin fraction.

Eventhough the mechanism of enhancement of enzymatic activity by saponin fraction was not clear, 5 restriction endonucleases AccI, XhoII, SalI, HincII, and BamHI were observed to cut more pBR322 at the appropriate concentration of ginseng saponin fraction (10^{-1}%-10^{-2}%).

Such results suggest that ginseng saponin fraction could save reaction time and then spare on the amount of the enzymes to be used, and activities of the rest of endonuclease would be enhanced.

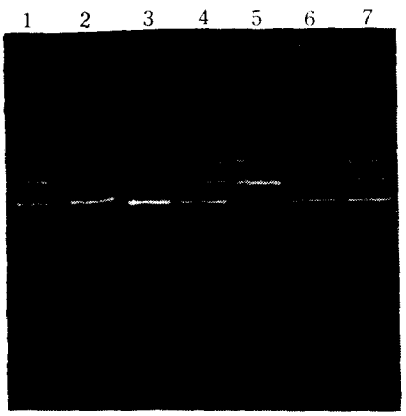

Figure 5. Agarose gel electrophoresis restriction pattern of pBR322 after the action of BamHl for 15 min incubation. Lane 1 is control, lanes from 2 to 6 are products digested by BamHl in the presence of the saponin fraction (10^{-1}-10^{-6}%). The amount of pBR322 used as a substrate was 2 μg.

要 約

인삼 saponin분획에서 pBR322가 더 잘 절단되는 것이 사용된 효소의 시간과 양을 줄일 수 있다는 사실과 연관이 있음을 보이기 위해 본 연구를 시도하였다.

Acc I, Xho II, Sal I, 그리고 Hinc II와 같은 제한효소들은 10^{-1}%인삼 saponin분획에서 효과적으로 pBR322를 절단한다는 것이 관찰되었고 BamH I의 경우는 10^{-2}% 인삼 saponin 분획이 가장 효과적인 농도임이 관찰되었다.

이와같은 일련의 결과들은 인삼 saponin분획이 제한효소에 의해 짧은 시간 안에 pBR322를 절단하는데 중요한 역할을 하는 것으로 사료된다.

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