Studying the Effect of Partially Purified *Arthrobacter*B1.Polysaccharide on Nucleosomal Distribution of HepG2 Tumor Cell Line

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Abstract

This study was designed to investigate the effect of partially purified polysaccharide extracted from *Arthrobacter*B1 on the DNA banding pattern of HepG2 tumor cell line *invitro*. The polysaccharide was extracted from *Arthrobacter* B1using phenol sulfuric acid methodwith a recovery yield of 181mg. Partial purification of polysaccharide was performed by using sepharose Cl-6B gel filtration chromatography and two peaks were obtained. Chemical analysis involving the estimation of carbohydrate and protein contents which showed that the large first peak contained the higher carbohydrate contents (30.1%) as compared with the crude polysaccharide 15.9% with low protein constituents (5.7%).The effect of *Arthrobacter* B1polysaccharide on HepG2 tumor cells was examined by studying whether bacterial polysaccharide participating in affecting the structure of tumor cell's DNA or not. Results indicated that only intact nucleosomal DNA bands were separated on the gel for the untreated cells as compared with the banding pattern of the colchicine treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated on the gel following electrophoresis process. Such pattern does not affected by the gradual increase in MNase concentration. On the other hand, the DNA banding pattern for the cells treated with *Arthrobacter* B1 polysaccharide showed significant effect by the separation of nucleosomal DNA, in which mononucleosomes, dinucleosomes and oligonucleosomes were clearly separated from intact nucleosomal DNA, and such effect was markedly appeared by increasing the concentration of MNase as compared with the cells treated colchicine.

Keywords**:** *Arthrobacter*, Polysaccharide, DNA, MNase.

Introduction

The ability of biological materials or synthetic compounds to affect the genetic material has been well established (1). Many studies appeared that bacterial preparations such as hole cells or cell wall possess marked inhibitory activity against progression and the development of tumor cells *in vitro* and *in vivo* (2). However, few studies reveled how bacterial cell wall preparation can affect the genetic materials of tumor cells.

Histones order eukaryotic DNA into structural units called nucleosomes, and histones consider being the major constituent of chromatin which are subjected to several different structural modification (3). The chromatin structure classified into euchromatin which lightly packed and relatively transcriptionally active and hetrochromatin, which is the packed and transcriptionally poor form of chromatin (4).

Structural modification of chromatin plays an important role in regulating the mechanism of gene expression converting euchromatin into more open and transcriptionally active (5).

Microbial polysaccharides may contributed in microbial such modification. Studies indicated that microbial polysaccharides were involved in several cellular processes such as molecular recognition, cell development, exert antioxidant, antiviral and antitumor activity (6).

Arthrobacter is a genus containing species of Gram positive bacteria, and its polysaccharides a represent a class of high-value polymers, that can interact with living cells displaying biological properties such as antioxidant, immunostimulant (7), and the ability to inhibit tumor growth and enhances immune function (8). Biological activity of the polysaccharides has attracted more attention recently in the biochemical and medical areas because of their immunomodulatory and

antitumor effects. The ability of bioactive polysaccharides and polysaccharide bound proteins to modulate so many important immune functions may be due to the structural diversity and variability of these macromolecules. For this reason this study conducted the use of the partially purified polysaccharide and test whether it will affect the banding pattern of HepG2 tumor cell line.

Materials and Methods

Source of bacteria, cell line and growth conditions

Arthrobacter sp. B1 was previously isolated from soil sample, which was examined microscopically, morphologically and biochemically (9). The sample was finally identified in Biotechnology Dept. laboratories –Al-Nahrain University. For biomass production, the isolate was grown in LB broth (10). HepG2 tumor cell line cells were kindly provided from Biotechnology Research Center, at Al-Nahrain University, cells were maintained by using RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium bicarbonate and 104 IU Penicillin G.

Extraction of *Arthrobacter* Polysaccharide

Arthrobacter cells were prepared by growing in a flask containing 25 ml of LB broth before incubation at 30 ºC for 18 hrs. The fresh culture was used to inoculate 3-3.5 L of LB broth suspended in a 500 ml conical flask filled with 200 ml broth. The inoculated flasks were incubated at 30 ºC for 24 hours with shaking at 150 rpm. Cultures were centrifuged (3000 rpm, 15 min), and pellets were washed twice with phosphate buffer. Cells were suspended in phosphate buffer containing 0.5% formalin (pH=7.2) before keeping at 4ºC for18 hrs. After that, the cells were centrifuged (3000 rpm, 15min) and washed with phosphate buffer. Finally, cells were dried to one-tenth of the original sample size by using cold acetone (11). The dried cells then used for polysaccharide extraction using a procedure described by Chandan and Fraser, (12).

Partial Purification of *Arthrobacter* Polysaccharide

This was performedby gel filtration chromatography, the gel was prepared according to the manufacturer instruction in which the gel was washed several times with 0.025 M phosphate buffer pH: 7.2. Gas bubbles were degassed using vacuum pump. Then the gel was slowly poured into the inner side of the column (3×50 cm) and equilibrated with the phosphate buffer (0.025 M, pH: 7.2) with a flow rate 30 ml/hour. Crude polysaccharide (30 mg/5 ml) was added gently through the sepharose Cl-6B column that has been previously equilibrated with phosphate buffer using a pasture pipette. Fractions have been collected at a flow rate 30 ml/hour and with fraction size of 3 ml/tube.

The collected fractions were examined by measuring the absorbance at 280 nm for detecting the contaminated proteins within fractions (14), estimating the carbohydrate concentration according to method described by Dubois *et al* (15), measuring the absorbance at 260 nm for detecting the nucleic acids (16) and determined the protein concentration according to the method described by Bradford (17).

Treatment of HepG2 tumer cell with polysaccharide and colchicine

HepG2 tumor cell were cultured in 125 ml flasks containing 20 ml of complete RPMI-1640 medium for 48 hrs at 37°C under 5% CO_2. After the formation of the confluent monolayer, flasks were treated as follows.

a) Five flasks were treated with colchicine at a final concentration of 50 µg/ml, then flasks were incubated at 37°C for 24 hrs under 5% CO_2.

b) Five flasks were treated with the partially purified *Arthrobacter* polysaccharide at a final concentration of 1 mg/ml. Flasks were incubated at same above condition.

c) Five flasks were used as a control (treated) and incubated for further 24 hrs at 37 °C for 24 hrs with 5% CO_2.

After incubation, the treated and untreated plates were mitotically shack-off by hard tapping the plates on the bench, in order to deattach the cells from the plates and suspended in the medium. To ensure the

complete cell deattachment, 2-3 ml trypsin were applied for few minutes. Cells (both treated and untreated) were fixed by 10% formaldehyde for 10 min, then the fixation was stopped by adding 5% glycine (2.5 M). cells were spined in portable centrifuge (1500 rpm) for 10 min, supernatant was discard and the pellets were suspended in 3ml of MNase cleavage buffer, and cell concentration was adjusted to $4*10^6$ cells/ml using the Haemocytometer. Cells, then, were flash freezed and kept at -20 °C until use (18).

MNase cleavage and cell digestion (19)

One hundred µl of cell suspension from each colchicine treated cells, polysaccharide treated cells and untreated cells were used.MNase reaction of 0, 5, 7.5 and 10 µl/ml MNase in MNase cleavage buffer was performed for 4 min at 37°C. The Reaction was stopped by adding 20µl of 0.5M EDTA, followed by adding 100µl MNase cleavage buffer for each tube. MNase - digested cells were then treated with proteinase K to a final concentration of 0.2 mg/ml and SDS of final concentration of 1%. The tubes were overnight incubated at 65°C, then DNA was extracted (20) and DNA banding patterns were inspected following gel electrophoresis using gel documentation system (Bio-Rad Germany).

Results and Discussion

Polysaccharide Extraction

The adopted method is a combination between the application of pressure and hot water for bacterial cell destruction and hydrolytic enzyme treatment for protein and nucleic acid removal. LB broth has been effectively employed for cultivation of *Arthrobacter* B1isolates besides the supplementation of 10mM glucose results in higher biomass yield and recovery of polysaccharide (20 gram weight bacteria) (21). Results indicated that, the polysaccharide recovery from the *Arthrobacter*sppB1 was 181 mg dry weights following lyophilization.

Partial Purification of *Arthrobacter* spp polysaccharide

Sixty fractions were collected and polysaccharide was recovered by detecting the carbohydrate contents for each fraction according to the method of Dubois (15) at 490 nm, also the contents polysaccharide-associated proteins were detected at 280 nm. Results in Fig. (1) shows the separation of two peaks, one large mostly consisting of carbohydrates and the other is small and both peaks, were followed by protein peaks. Chemical of the large peak showed that the percentage of carbohydrate increased from 15.9% for the crude polysaccharide to 30.1%.

According to Grobben (22) polysaccharide have different molecular weight, so the purification of *Arthrobacter* B1 polysaccharide through sepharose Cl-6B that showed high molecular weight can be easily separated (fractions between 14-21) from lower molecular weight impurities. Chemical analysis of the partially purified polysaccharide in the large peak (peak 1) was determined. Chemical analyses involve determination of carbohydrate, protein and nucleic acid contents for the active fractions from 14^{th} to 21^{th} appear in the large peak. Results indicated that the percentage of carbohydrates was 31.1% in the large peak.

Reports were revealed the ability to obtain 2 fraction or peaks after loading the microbial polysaccharide on different charomatographic columns (ion exchange or gel filtration) in which the sugar content in the first large fraction was measured to be around 23% (23, 24). Robjin (25) reported that the overall percentage of carbohydrates present in *Lactobacillus* structure polysaccharide is 65% while a percentage of 84% was reported by Vijayendra (26) for the *Leuconostoc* polysaccharide. These differences in calculations are generally attributed to the types of bacterial species from which polysaccharide was extracted, method of extraction and purification process.

On the other hand, the protein contents were also investigated and results showed that the protein percentage was 5.7% peak 1. In addition the application of nucleases enzymes results in 0% of nucleic acids after gel filtration purification of polysaccharide.

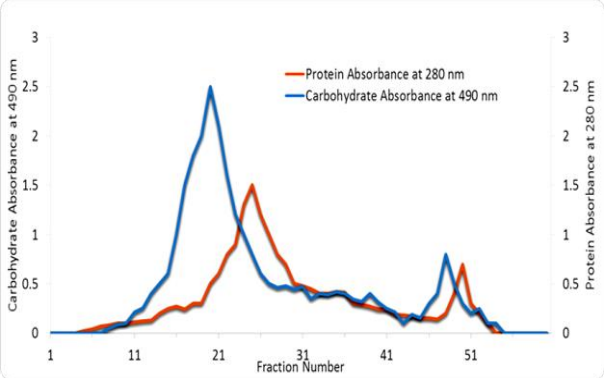

Fig.(1) Purification of polysaccharide from Arthrobacterspp. by gel filtration chromatography (Sepharose Cl-6B) column (3x50 cm) equilibrated with 0.025M phosphate buffer pH 7.2, flow rate 30ml/hour.

Effect of Partially Purified *Arthrobacter* B1 Polysaccharide on the DNA of HepG2 cell Line

The effect of *Arthrobacter* B1 polysaccharide on HepG2 tumor cells was examined by studying the whether bacterial polysaccharide affect the structure of tumor cell's DNA. Fig. (2) shows the differences in DNA banding pattern between the untreated cells and those cells treated with colchicine. Results indicated that only intact nucleosomal DNA bands were separated on the gel for the untreated cells as compared with the banding pattern of the colchicine treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated on the gel following electrophoresis process. In addition in both cases the gradual increase in MNase concentration did not significantly affect the DNA digestion.

Colchicine arrest the cell cycle at metaphase stage, this phase characterized by disappearing of nuclear envelop and the DNA being more condensed and packed into chromosomes (27). Metaphase chromosomes are widely used as comparing control in many experiments related to nucleosome partitioning via Mnase digestion (28). In addition, the metaphase chromosomes contains very fewer non-histone protein in their structure in which the remaining metaphase scaffold mainly consist of topoisomerase which has a role in stabilizing the bases of DNA loops of metaphase chromosomes (28). Moreover recent study indicated the cytogenetic effect of colchicine (10µg/ml) on the chromosomes of normal lymphocyte cells. The observations of the researchers were correlated between the exposure period and the length of the chromosomes, in which extended periods of exposure leads to more short and condense chromosome, in contrast short exposure period of several hours gave longer chromosomes (29).

In Fig.(3) the DNA banding pattern shows that *Arthrobacter* B1polysaccharide significantly affects the separation of nucleosomal DNA, in which mononucleosomes, dinucleosomes and oligonucleosomes were clearly separated from intact nucleosomal DNA, also by gradual increasing the concentration of Mnase yield more separation as compared with the cells treated colchicine. According to this result, it was suggested that *Arthrobacter* polysaccharide participate in tumor cell lysis by induction of DNA fragmentation, which is a characteristic feature of cell apoptosis.

Bacterial polysaccharide have been received an increasing attention in research through the last few decades. The roles of polysaccharides in biological systems were involved in several cellular processes, such as molecular recognition, cell development and differentiation, and cell–cell interaction. Many polysaccharides exerted potent antioxidant, anticoagulant, antithrombotic and antiviral activities and they are also known to inhibit some tumor development (8).

Studies suggested that the flow cytometry analyses of polysaccharide from different sources causes' cancer cell cycle arrested at the G0/G1 or the G2/M check points (30). In addition microbial polysaccharides can cause the tumor cells to undergo apoptosis by regulating signaling molecules such as NF-κB, besides to the change of Ca2+ concentration or can induced tumor cells to apoptosis. Moreover polysaccharides can indirectly inhibit the growth of tumor cells by improving the immune functions or inhibiting DNA synthesis (30). Shi (6) were reported that treatment of human gastric carcinoma cells with polysaccharide being chemically modified with sulphate group induced cell

apoptosis through DNA fragmentation which characterized by gel electrophoresis as distributed nucleosomal ladder of fragmented chromatin. It's very important to know that identification of nucleosome distribution and chromatin accessibility profiles provides a new set of measurements for the biological activity and/or antitumor activity of natural and chemical substances on the tumor cells. Many studies defined the role of bacterial polysaccharide extracted from their cell wall as either antitumor agent or as immunomodular (31), however little is known about how polysaccharide affect the genetic material and the gene expression of the tumor cells via chromatin remodeling which responsible for altering the position and density of nucleosomes and in turns it may allow genes to be transcriptionally active or not. Such strategy was adopted by treating immune cells with bacterial polysaccharides determine the genetic changes with respect to immune response, the resulted changes indicated that large number of affected promoter regions confirms that polysaccharide signaling in human macrophages induces a massive redistribution of nucleosome at many immune genes (32).

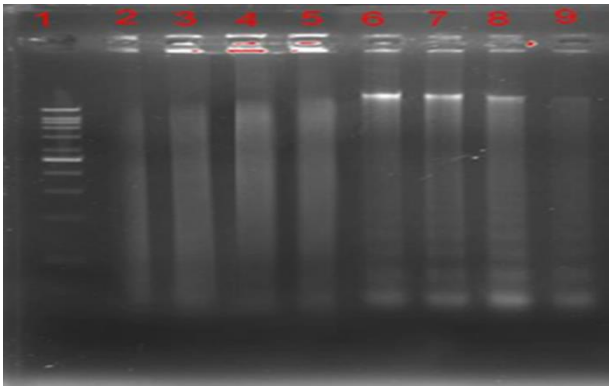

Fig.(2) DNA extracted from HepG2 cells, lane1 represent marker, lane 2 DNA from untreated cells undigested with MNase, lane3 DNA from untreated cells digested with 5 µl MNase, lane4 DNA from untreated cells digested with 7.5 µl MNase, lane5 DNA from untreated cells digested with 10 µl MNase, lane 6 DNA from cells treated with colchicine undigested with MNase, lane 7 DNA from cells treated with colchicine digested with 5 µl MNase, lane 8 DNA from cells treated with colchicine digested with 7.5 µl MNase and lane 9 DNA from cells treated with colchicine digested with 10 µl MNase.Electrophoresis was performed at 100 volts for 45min.

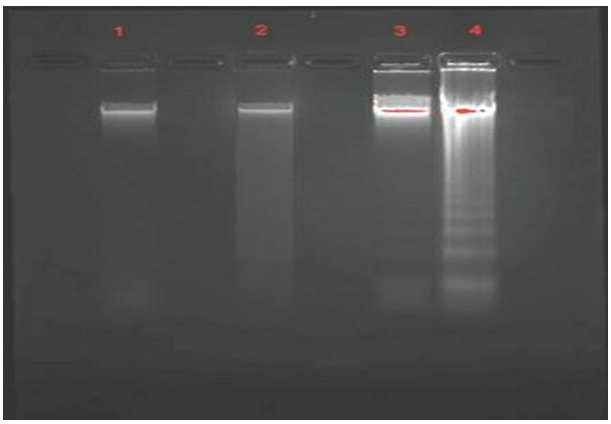

Fig.(3) DNA extracted from HepG2 cells treated with Arthrobacter B1 polysaccharide, lane 1 cells undigested with MNase, lane 2 cells digested with 5 µl MNase, lane 3 cells treated with 7.5 µl MNase and lane 4 cells digested with 10 µl MNase.

Conclusions

Only intact nucleosomal DNA bands were separated on the gel for the untreated HepG2 cells as compared with the banding pattern of the colchicineand Arthrobacter B1 polysaccharide treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated. Nucleosomal DNA bands pattern of both untreated and colchicine treated DNA does not affected by the gradual increase in MNase concentration, but nucleosomal DNA bands pattern of DNA treated with polysaccharide in which the separation of nucleosomes became more clear by increasing the concentration of MNase.

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الخلاصة

صممت هذه الدراسة للتحقق من تاثير متعدد السكريات البكتيري الموجب لصبغة كرام و المستخلص والمنقى جزئيا من بكتريا *Arthrobacter* sp. على نمط حزم الحامض النووي (الدنا) لخطوط الخلايا السرطانية في الزجاج. استخلص متعدد السكريات من الغلاف الخارجي لبكتريا *Arthrobacter* B1 اعتمادا على الطريقة التي وصفت بواسطة Chandan و Fraser (١٩٩٤) حيث كان عائد الاستخلاص هو ١٨١ ملغم. كما اظهرت نتائج التحليل الكيميائي ان المحتوى الكاربوهيدراتي والبروتيني ١٥,٩% و ١,٩% على التوالي. تم اعتماد التنقية الجزئية لمتعدد السكريات بواسطة استخدام كروماتوكرافيا الترشيح الهلامي باستخدام هلام (SepharoseCl- B6) وبعد التنقيه تم الحصول على قمتين و بعد تحديد الخواص الكيميائيه و التي تنطوي على تقدير المحتوى الكاربوهيدراتي والبروتيني اظهرت النتائج بأن القمة الاولى كانت ذات محتوى كاربوهيدراتي عالي ٣٠,١% مع مكونات بروتينيه قليلة ٥,٧%.

درس تأثير مستخلص متعدد السكريات المنقى جزئيا من بكتريا *Arthrobacter* على خط الخلايا السرطانية HepG2 وذالك بدراسة في ما اذا لمتعدد السكريات البكتيري تاثير في تركيب الحامض النووي للخلايا السرطانيه ام لا. اشارت النتائج الى انه فقط حزم الدنا النيوكليوسومي الكاملة قد فصلت في الهلام لدنا الخلايا الغير معاملة بالمقارنه مع نمط حزم الحامض النووي للخلايا المعاملة مع الكولجسين حيث بالاضافة الى الدنا النيوكليوسومي الكامل تم الحصول على فصل للنيوكليوسوم الاحادي, الثنائي والنيوكليوسوم المتعدد القليل في الهلام بعد عملية الترحيل الكهربائي. أن مثل هذا النمط لم يتأثر بالزيادة التدريجية لتركيز MNase. من جانب اظهر نمط حزم الحامض النووي للخلايا المعاملة مع متعدد السكريات المستخلص من *Arthrobacter* B1 تأثيرا معنويا من خلال فصل نيوكليوسوم الحامض النووي الاحادي، الثنائي و المتعدد القليل بصورة واضحة عن نيوكليوسوم الحامض النووي الكامل حيث ان مثل مثل هذا التاثير كان ظاهراً بصورة واضحة مع زيادة تركيز MNase بالمقارنه مع الحامض النووي للخلايا المعامله بالكولجسين.