Structural Characterization and Biological Activity of Acidic Polysaccharide Fractions Isolated from *Bacillus polymyx*a NRC-A

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**Abstract:** The heteropolysaccharides coded as AP-I, AP-II and AP-III were obtained from the crude extracellular polysaccharide of *Bacillus polymyx*a NRC-A by eluting from DEAE-cellulose column and Sephadex G-150 column. Their chemical structure and molecular weights were characterized by IR, GC-MS, periodate oxidation-Smith's degradation and gel permeation chromatography (GPC). These polysaccharides comprised mainly glucose, mannos, rhamnos and glucouronic acid in the ratio of (3.00: 2.10: 1.40: 1.00), (3.40: 2.52:1.00: 2.11) and (4.11: 2.68: 1.00: 3.00) in AP-I, AP-II and AP-III, respectively. The antioxidant activities of the AP-I, AP-II and AP-III were evaluated by DPPH radical scavenging. The order effectiveness of polysaccharide fractions in inhibiting free radicals was as follows: AP-III>AP-II> AP-I. The effects of the molecular weight and uronic acid content of the polysaccharide fractions on the improvement of the bioactivities appeared to be significant. In addition, these native and modified fractions still determined as *in-vitro* anticoagulant activity. The modified fractions showed high anticoagulant activity than the native fractions.

**Keyword:** Exocellular polysaccharide (EPS), *Bacillus polymyx*a NRC-A, GC-MS, periodate oxidation, Smith-degradation, antioxidant, anticoagulant.

**INTRODUCTION**

Microbial exopolysaccharides are produced by various genera of bacteria and yeast[1,2]. Many of these products have been shown to have a wide variety of applications in food, pharmaceutical and oil industries[3]. Some microorganisms belonging to *Bacillus* species have been known to produce extracellular polysaccharides, such as cellulose[4], levan[5], mannan[6] and acidic polysaccharides[7]. But Mitsuda's[8] produced the EPS composed of glucose, mannos and glucouronic acid by *Bacillus polymyx*a No. 458. The viscosity of the EPS isolation was 5-8 times higher than that of locust bean gum. A previous study[9] showed that an extracellular acidic polysaccharide produced by a new isolated microorganism, designated as *Bacillus polymyx*a, reduced the cholesterol level in serum and liver of hypercholesterolemic rats[10]. Recently, some plant polysaccharides such as pectin, guar gum, and sodium alginate have attracted attention for their hypcholesterolemic effect on experimental rats. In the course of study, it has been found that some strains of *Bacillus* sp. were capable of producing new types of acidic heteropolysaccharides. Reactive oxygen species (ROS), capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age[11,12]. The polysaccharides have been demonstrated to play an important role in dietary free-radical scavenging for oxidative damage prevention. There is an increasing evidence indicating that the reactive oxygen species produced by sunlight, ultraviolet light, ionizing radiation, chemical reaction and metabolic process have a wide variety of pathological effects. These effects might be DNA damage, carcinogenesis, and cellular degeneration related to aging[13,14]. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases[15]. Recently, many natural resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods. Some alga polysaccharides have been demonstrated to play an important role as free-radical scavengers in vitro and as antioxidants for the prevention of oxidative damage in living organisms[16]. Their activity depends on several structure parameters such as molecular weights, type of sugars and glycosidic branching[17,18]. This work was conducted to study the possible isolation, purification, fractionation and structure features of the acidic polysaccharide produced by isolated *Bacillus polymyx*a NRC-A, in addition to the, biological activities of these fractions.
MATERIALS AND METHODS

Isolation and Identification of Bacteria: Polysaccharide-producing bacteria were isolated from soil samples collected from different zones. Isolates were obtained by serial dilution plating on a seed media containing (g/l), glucose 20.0; CaCO₃ 1.0; NH₄NO₃ 0.8; K₂HPO₄ 0.6; KH₂PO₄ 0.05; MgSO₄·7H₂O 0.05; MnSO₄·4H₂O 0.1 and Yeast extract 0.1, at 30°C [19]. A total of about 100 colonies were isolated, and the polysaccharide-producing bacteria were screened for their ability to produce polysaccharide this was based on colony morphology (mucous andropy). A mucous colony was isolated and identified as Bacillus polymyxa NRC-A. Metabolic characterization was carried out in Biolog GP2 Micro Plates TM according to the instructions of the manufacturer (Biolog, Hayward CA, USA) and evaluated with Microlog3 Software.

Isolation and Purification of Polysaccharide: Bacillus polymyxa NRC-A was grown in a liquid seed media but using the following concentrations (60.0 g glucose; 4.0 g CaCO₃ and 2.0 g NH₄NO₃) by the method of [20] under shaking condition; 150 rpm for 3 days. The culture broth was diluted with water and centrifuged at 5000 rpm for 20 min (Sigma-Laborzentrifugen, 2K 15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight at 4°C and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times (3 x 1L). The supernatant was completed to four-volumes with ethanol 95% and left overnight at 4°C. The precipitate was separated by centrifugation at 5000 rpm, washed twice with acetone and dehydrated by ether. The crude polysaccharide was dissolved in 0.03 M Na₂SO₄, and precipitated by cetlypyridinium chloride. The precipitated CPC-Complex was collected by centrifugation, washed several times with ethanol, and dissolved in 10% NaCl. The solution was filtered, dialyzed, and concentrated to a small volume. The purified polysaccharide was precipitated by ethanol, yielding a white powder of an acidic polysaccharide [21].

Anion-exchange Chromatographic Method: The acidic polysaccharide (100 mg in 5 ml water) was subjected to anion exchange chromatography on a column (2.0 x 40cm) of DEAE-cellulose, pre-equilibrated using phosphate buffer 0.1 M and eluted with continuous gradient from 0.0 to 4.0 M NaCl in the same buffer [21]. Fractions (5 ml) were collected and an aliquot (0.1 ml) was tested for total carbohydrate by the phenol-H₂SO₄ method using Spectrophotometer UV-2401PC Shimadzu [22]. The respective polysaccharide fractions were pooled and dialyzed overnight against deionized water and finally lyophilized.

Gel-filtration Chromatography: Each fraction was subjected to gel permeation chromatography on a column (2.6 x 50 cm) of SephAdex G-150, which was pre-calibrated with dextrans (Fluka), of known molecular weight. The void volume was determined using dextran blue. The column was equilibrated and was eluted with 0.1 M NaCl. An aqueous solution of sample (50 mg) was dissolved in 2.0ml of 0.1 M NaCl and loaded onto the column bed. Fractions (5.0 ml) were collected and tested for total carbohydrate by the phenol-H₂SO₄ method [22].

Molecular Weight Determination: The average molecular weights of AP-I, AP-II and AP-III were determined by a gel-chromatographic technique. Standard dextran 2000000, 700000, and 40000 (Fluka) were passed through a (2.6 x 50 cm) SephadexG-150 column, and then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volumes of AP-I, AP-II and AP-III were plotted in the same graph, and the molecular weights were determined [23].

Determination of Polysaccharides Sugar Composition: The polysaccharides AP-I, AP-II and AP-III were hydrolyzed with 2.0 M triflouroacetic acid (TFA) and the tubes were heat-sealed. Hydrolysis was carried out at 105°C for 4 h in an oven. After the hydrolysis, the acid was removed by evaporation on a water bath at a temperature of 40°C and co-distilled with water (3 x 5 ml) [24]. The purified hydrolyzates (20µl) were analyzed by HPLC according to El-Sayed's [25].

Infra Red Spectroscopy: The polysaccharides were also characterized using a Fourier transform infrared in Bucker Scientific 500-IR spectrophotometer. The dried polysaccharides were grounded with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 400-4000 cm⁻¹ [24].

Periodate Oxidation-Smith Degradation: Samples of AP-I, AP-II and AP-III (16 mg) were added separately to 50 ml NaIO₄ 0.1 M solution in round bottom flasks, and the mixtures were kept at 4°C in the dark [27]. Aliquots (2 ml) were removed after different intervals of time and consumption of periodate and HCOOH products were determined [28,29]. Ethylene glycol (2 ml) was added, and then the experiment of periodate oxidation was over. The solutions were dialyzed against tap water and distilled water for 48 h, respectively.
The polyaldehyde was reduced with excess of NaBH₄ at room temperature overnight. The reaction was terminated by addition of ice cold acetic acid (4 N). The solutions were again dialyzed as described above, and lyophilized[31]. The resulting polyalcohol was hydrolyzed with HCOOH 90% for 5 h. The sugars and sugar alcohols were analyzed by HPLC according to El-Sayed’s[33].

**Methylation Analysis:** The native polysaccharide fractions (AP-I, AP-II and AP-III) and their carboxyl-reduced by the method of Taylor[34] (AP-IR, AP-IIR and AP-IIIR) were methylated separately using the method delete[35]. The methylated products were extracted by CHCl₃. The product was then hydrolyzed with 90% HCOOH (5ml) for 5h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with NaBH₄ (24 mg), and acetylated with pyridine and acetic acid[33]. The alditol acetates of the methylated sugars were analyzed by GC-MS Finnegan SSQ-7000 instrument using a fused silica capillary column (DB-5, 0.25 mm ID, 30 m). A temperature programming of 60-280°C increased by 4°C/min was maintained for the analysis. Ionization potential was 70ev and mass range (m/z) was 40-4000amu. Helium was the carrier gas used. Qualitative and quantitative identification of the methylated sugars were determined by comparing retention time and mass fragmentation patterns with those of the available authentic data base.

**Measurement of Anti-oxidation Activity:** For the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 10 mg (in 100 µl saline) each sample was added to 900 µl of DPPH solution (Freshly prepared at a concentration of 10⁻⁴ M) and the mixtures were mixed for 10 sec at ambient temperature 22°C. The decrease in absorption at 515 nm was measured against a blank of ethanol without DPPH in 1-Cm quartz cells after 10, 20, 30, and 60 min of mixing using Spectrophotometer UV-VIS 2401PC Shimadzu[36]. Antiradical action toward DPPH radical was estimated from the difference in absorbance with or without sample (control) and the percent of inhibition was calculated from the following equation: Inhibition % = (Abs. of control – Abs. of test sample) x 100/ Abs. of control

The samples of each polysaccharide fractions were analyzed and the main values as well as the (S.E) were given.

**Anti-coagulant Activity:** Venous blood was collected directly into a vessel containing one tenth volume 3.2% trisodium citrate. The mixture was immediately agitated, by gentle inversion, centrifuged at 2000 rpm for 10min. The canary yellow plasma was pooled[35]. Anti-coagulant activities of the various original and modified polysaccharide fractions were determined[36] and compared with reference sodium heparin.

**RESULTS AND DISCUSSIONS**

**Isolation, Purification and Composition of EPS:** After *Bacillus polymyxa* NRC-A was grown for three days, the bacterial cells were separated by centrifugation, and the crude polysaccharide was precipitated by ethanol from the supernatant. The crude polysaccharide was dissolved in sodium sulfate, which was further fractionated by treatment with cetlypyridinium chloride (CPC), to give an acidic polysaccharide fraction (11.3gl⁻¹) and a neutral polysaccharide (3.63gl⁻¹) which was kept for further study. The acidic polysaccharide was purified by repeated fractional precipitations with ethanol, and upon chromatography on DEAE-Cellulose (Pharmacia) gave three fractions: AP-I, AP-II and AP-III (eluted with 0.01 M phosphate buffer containing 0.0 to 4.0 M NaCl) (Fig. 1). The three fractions AP-I, AP-II and AP-III were purified by gel filtration chromatography on Sephadex G-150 column (2.6 x 50 cm) showing a single and symmetrical peak, indicating that the each fraction had been purified to homogeneity (Fig. 2-4). The molecular weights of the polysaccharide fractions AP-I, AP-II and AP-III were determined by a gel filtration technique using different Dextran markers passing through a Sephadex G150 column (2.6 x 50 cm), and they were found to be 130 kDa, 110 kDa and 80 kDa, respectively. Analysis of their acid hyrolyzates by HPLC indicated the presence of glucose, mannose, rhamnose and glucouronic acid in the ratios of (3.00: 2.10: 1.40: 1.00), (3.40: 2.52:1.00: 2.11) and (4.11: 2.68: 1.00: 3.00) in AP-I, AP-II and AP-III, respectively. This indicates that these acidic...
Table 1: Molecular weights and monosugar molar ratios of fractions polysaccharide obtained from chromatographic column.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>M Wt (KDa)</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Rhamnose</th>
<th>Glucuronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-I</td>
<td>130</td>
<td>3.00</td>
<td>2.10</td>
<td>1.40</td>
<td>1.00</td>
</tr>
<tr>
<td>AP-II</td>
<td>110</td>
<td>3.40</td>
<td>2.52</td>
<td>1.00</td>
<td>2.11</td>
</tr>
<tr>
<td>AP-III</td>
<td>80</td>
<td>4.11</td>
<td>2.68</td>
<td>1.00</td>
<td>3.00</td>
</tr>
</tbody>
</table>

Table 2: Molar ratios of sugar alcohols and immune sugar in the Smiths degradation hydrolysate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sugar alcohols</th>
<th>Sugar immune</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythretol</td>
<td>Erythritic acid</td>
</tr>
<tr>
<td>AP-I</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>AP-II</td>
<td>3.8</td>
<td>0.2</td>
</tr>
<tr>
<td>AP-III</td>
<td>4.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 3: Linkage analysis of the constituent sugars of the fractions AP-I, AP-II and AP-III and their carboxyl- reduced fraction AP-IR, AP-IIR and AP-IIIR.

<table>
<thead>
<tr>
<th>Methylated alditol acetates</th>
<th>Mode of linkage</th>
<th>Molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,6-Me₃-Glc</td>
<td>Glc (-4) Glc (1-4)</td>
<td>AP-I</td>
</tr>
<tr>
<td>2,3-Me₂-Glc</td>
<td>Glc (1-2) Glc (1-4)</td>
<td>3.4</td>
</tr>
<tr>
<td>2,3,4,6-Me₄-Glc</td>
<td>Glc (1-4) Glc (1-4)</td>
<td>2.0</td>
</tr>
<tr>
<td>2,4-Me₂-Rha</td>
<td>Rha (-3) Rha (1-3)</td>
<td>1.0</td>
</tr>
<tr>
<td>2,3,6-Me₃-Man</td>
<td>Man (1-4) Man (1-4)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Fig. 2: Gel filtration for the chromatography of AP-I on Sephadex G-150 (2.6x50cm i.d.), the column was equilibrated with 0.1 M NaCl at flow rate 0.5 ml/min and 5ml fraction.

Fig. 3: Gel filtration for the chromatography of AP-II on Sephadex G-150 (2.6x50cm i.d.), the column was equilibrated with 0.1 M NaCl at flow rate 0.5 ml/min and 5 ml fraction.

Polysaccharide fractions have the same chemical composition in spite of the difference in the glucuronic acid ratio (see table 1). The FT-IR Spectra of AP-I, AP-II and AP-III showed adsorption bands at 704.9, 788.5, 845.7, 891.7, 1069.7, 2921.7 and 3423.3 cm⁻¹ in the IR spectrum (Fig. 5). The band at 891.7 cm⁻¹ was ascribed to be β-type glycosidic linkages in the polysaccharide. The bands at 891.7 and 932.1 cm⁻¹ were characteristic of (1→4)β-glycosidic linkages. The broad band at 16391 cm⁻¹ was due to bound water. The IR spectra indicated the presence of β-type glycosidic linkages in the AP-I, AP-II and AP-III.

Periodate Oxidation and Smith Degradation: The AP-I, AP-II and AP-III were submitted to periodate oxidation, borohydride reduction and hydrolysis by heating with 90% HCOOH for 5 h (Smith degradation). The AP-I, AP-II and AP-III consumed 0.76, 0.73 and 0.84 mol of oxidant per mol of glycosyl residue, respectively and a small amount of formic acid was consumed.
Gel filtration for the chromatography of AP-III on Sephadex G-150 (2.6x50cm i.d.), the column was equilibrated with 0.1 M NaCl at flow rate 0.5 ml/min and 5 ml fraction. liberated. The HPLC analysis of the Smith's degraded polysaccharides, the erythritol, erythric acid, and glycerol in addition to rhamnose was determined. The molar ratios of these sugar derivatives are shown in (Table 2).

Erythritol was produced from (1--4)-glycosidic linkages of glucose and/or mannose. While, erythric acid was liberated from (1--4)-glycosidic linkages of glucuronic acid. Glycerol detected in the polyalcohol hydrolyzate indicated the presence of (1--4) glycosidic linkages glucose and/or mannose at the non-reducing end. The disappearance of glycemic acid through the hydrolysis of the polyalcohol means that glucuronic acid did not exist at the non-reducing end, but probably was found inside the backbone[18,19]. The appearance of rhamnose as the immune unit indicated the presence of some (1--3) glycosidic linkages rhamnose[20,21]. Thus, the periodate oxidation and Smith's degradation study confirmed the mode of linkages, which agrees with presence of the IR bands[22].

**Linkage Analysis:** To obtain some definite information on the mode of glycosidic linkages of these sugar residues, the polysaccharide fractions were methylated and then the methylated sugars were converted to the corresponding acetylated derivatives. As shown in (Table 3) glucose, mannose and glucuronic acid residues in the main chain of the polysaccharide are linked mainly through (1--4) and (1--6) glycosidic linkages, as indicated by the presence of 2,3,6 tri-O-methyl glucose, 2,3,6 tri-O-methyl mannose, and 2,3 di-O-methyl glucose.

**Table 4:** Scavenging effect of polysaccharide fractions during DPPH test as measured by changes in absorbance at 515 nm.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-I</td>
<td>19.166±0.785</td>
<td>21.796±1.075</td>
<td>29.689±0.243</td>
<td>32.906±0.496</td>
</tr>
<tr>
<td>AP-II</td>
<td>19.583±0.420</td>
<td>23.742±0.730</td>
<td>39.246±0.714</td>
<td>43.276±0.190</td>
</tr>
<tr>
<td>AP-III</td>
<td>18.229±0.445</td>
<td>27.933±0.245</td>
<td>59.302±0.379</td>
<td>63.011±0.478</td>
</tr>
<tr>
<td>Dextran</td>
<td>14.193±0.358</td>
<td>17.460±0.253</td>
<td>21.542±0.528</td>
<td>21.970±0.485</td>
</tr>
</tbody>
</table>

* Values are means ± S.E of three determinations.

**Fig. 5:** Infrared spectra of acidic polysaccharide fractions AP-I, AP-II, and AP-III isolated from B. polymyxa NRC-A
In addition, the presence of 2,4 di-O-methyl rhamnose suggests that rhamnose was present in the main chain and linked mainly by (1-3) glycosidic linkages.

The presence of 2,3,4,6 tri-O-methyl glucose indicated that the glucose may be found at the non-reducing end. As shown the polysaccharide has a highly branched structure and the side chains are terminated with glucose. In addition, the presence of 2,3 di-O-methyl glucose suggests that some of the (1-4) glycosidic linkage glucose residues might come from a branch point. As regard the glycosidic linkages of glucouronic acid residues, the methylated carboxyl-reduced polysaccharides fractions gave about two-fold molar ratios of 2,3,6-tri-O-methyl glucose than native polysaccharide fractions, indicating that glucouronic acid residues are linked mainly by (1-4) glycosidic linkage. These agree with appearance of glycerol in smith degradation results. These results agreed with the appearance of rhamnose as immune units in the Smith's degradation results.[7,24].

Radical Scavenging Ability: Using the same per-weight basis the antiradical performance of polysaccharide fractions with respect to DPPH radicals was measured and compared. The order of effectiveness of polysaccharide fractions in inhibiting free radicals was as follows: AP-III>AP-II>AP-I. Table 4 shows that during the test periods, AP-III had the highest radical scavenging activity, followed by AP-II. After 60 min incubation, 63.01% of DPPH radical were quenched by fraction AP-III, followed by fraction AP-II which was able to quench 45.3%. Surprisingly, inhibition of DPPH radicals was only 21.9% pure dextran (Sigma) was assayed.

The results suggested that the molecular weights of polysaccharide fractions played an important role on their bioactivity. In the study of acidic polysaccharide, we obtained three fractions, and found they exhibited antioxidant ability depending on the uronic acid content. With increased of the uronic acid content, antioxidant activity of the three fractions increased. The existence of uronic acid might affect the physico-chemical properties of the polysaccharides and hence their bioactivities. In this study, found that both uronic acid content and molecular weight of acidic polysaccharide fractions could play on important role in the antioxidant activity. Among the acidic polysaccharides, a relatively low molecular weight and a high uronic acid content appeared to increase the antioxidant activity. The results were similar to Chen's reports that molecular weight was very important to the antioxidant activity of Green Tea polysaccharide fractions[43]. Polysaccharide extracts from mushroom[14] and Keisleriella sp. Y54108 exopolysaccharide[44] were also reported to have free-radical scavenging effects related to its affinity to the radical in the specific site. However, the mechanism of free-radical scavenging of polysaccharides is still not fully understood[45]. It is known that the phenolic compounds from the plant Pedicularis alashanica, such as phenylproponiod glycosides, may react with superoxide radical by a one-electron transfer mechanism or hydrogen abstraction mechanism to form the semiquinones. Therefore, the scavenging activity of their reduction may be related to the number of phenolic hydroxyl groups and the conjugated system[46]. However, it was not clear whether the mechanisms of radical scavenging by polysaccharide fractions were similar to that of plant phenolic compounds or not.

Anti-coagulant Activity: Plasma was coagulated in the blank-tube after 5 min and in the sodium heparin containing tube after 25 min. In experimental tubes, the highest anticoagulant activity was exhibited by AP-III native and AP-III polyalcohol as shown in (Table 5). The APIII containing negatively charged COO-glucouronic acid, also opening the pyranose rings by periodate treatment (Polyaldehyde and polyalcohol). Having the ability of binding Ca2+, therefore they prevent clotting formation[41,46,47]. These promising results can be regarded on initiatory steps towards the utilization of acidic polysaccharide and modified as future cheap sources for production of valuable drugs for treatments of blood coagulant.

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