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Characterization of Alginase and Elicitor-Active Oligosaccharides from *Gracilibacillus* A7 in Alleviating Salt Stress for *Brassica campestris* L.

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ABSTRACT: Alginase was purified from *Gracilibacillus* A7 and evaluated for its ability to produce elicitor-active oligosaccharides. The optimum conditions for the alginase reaction are as follows: temperature, 40 °C; pH, 8.0; alginate content, 0.3−0.7%; and the presence of Na^+ and Mg^{2+} metal ions. The degree of polymerization (DP) decreased as the reaction time of the alginase progressed, achieving values of 5.4 and 3.3 after 240 and 300 min, respectively. The relative root length (RRL) of the *Brassica campestris* L. increased with the addition of oligosaccharides with reduced DP values. The oligosaccharides with lower DP values are effective in reducing the effect of salt stress on the activity of the superoxide dismutase (SOD) and guaiacol peroxidase (POD), and oligosaccharides with moderate DP values can reduce the increase in lipid peroxidation activities (as malondialdehyde content) induced by salt stress. These results suggest that oligosaccharides may act as osmoprotective agents during the plant germination process.

KEYWORDS: *Gracilibacillus* A7, alginase, oligosaccharides, root elongation, salt stress

INTRODUCTION

Alginates are widely distributed in the marine environment and have been used as stabilizers, viscosifiers, and gelling agents in the food and beverage, paper and printing, biomaterials, and pharmaceutical industries.[1] Alginase enzymes have been found in various species of organisms in soil and marine environments, including invertebrate animals, protozoa, bacteria, and fungi.[2] The alginase enzyme catalyzes the degradation of alginate through β-elimination of the glycosidic bond. The production of various alginate oligosaccharides using alginase is believed to be important for the development of a more functional alginate to be used in future applications.[3]

Different types of alginases have exhibited a variety of processes of alginate degradation, which may be poly(M)lyase, poly(G)lyase, or a mixture of the two.[4,5] Some oligosaccharides derived from alginate have been shown to be elicitor-active with antibacterial and root growth-promoting effects.[6,7] The unsaturated end structure of oligosaccharides has been suggested to play a role in inducing tumor necrosis factor-α (TNF-α).[8] Alginase was also used to produce single-cell detritus from brown algal thalli to feed aquaculture animals.[9] The elicitor activities, such as plant growth promoting effect, are thought to be related to the molecular mass of the oligosaccharides.[6] The average molecular mass can be determined through chemical analysis and expressed as the degree of polymerization (DP).[6] Various oligosaccharides, including di-, tri-, and tetrasaccharides, can also be isolated through low-pressure gel permeation chromatography (LP-GPC), semipreparative strong-anion exchange (SAX), and fast-protein liquid chromatography (FPLC).[10]

To date, the capacity of alginase to produce functional oligosaccharides has been widely reported.[7,11] However, no research was conducted on the root growth promoting activity of oligosaccharides under salt stress, which is important in the applications of alginase and its products. Plants subjected to salt stress may produce reactive oxygen species (ROS), which can be scavenged by the antioxidant systems in plant tissues. Under conditions of salt stress, the activities of superoxide dismutase (SOD) and catalase (CAT) were inhibited, and peroxidase (POD) was induced.[12] Growing tissue demonstrated a greater stimulation of SOD and ascorbate peroxidase (APX) activity under saline conditions than mature tissue, whereas mature tissue showed up-regulated catalase stimulation.[13] At the final stages of germination, coinciding with radicle elongation, a second increase in superoxide ($O_2^{\bullet-}$), but not H_2O_2, occurred and was correlated with an increase in extracellular peroxidase (ECPOX) activity.[14] The antioxidant enzymes that directly react with ROS under salt stress include SOD, CAT, and POD, whereas lipid peroxidation, as measured by malondialdehyde (MDA) content, reflects oxidative damage to cell components.[15]

In our previous study, a novel alginate-degrading bacterium, *Gracilibacillus* A7, was isolated from seaweed compost and used in the composting process.[16,17] The results indicated that the seaweed-degrading product of *Gracilibacillus* A7 might be a useful fertilizer with plant growth promoting effects. However, the properties of the alginase from *Gracilibacillus* A7 and the mechanism of the functional degradation products have not been thoroughly elucidated. Characterizing the properties of the *Gracilibacillus* A7 alginase and further revealing the elicitor-active effect of

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degraded oligosaccharides on root growth under salt stress are important for the development of field applications for the bacterium *Gracilibacillus* A7 and its functional alginase.

MATERIALS AND METHODS

Purification of Alginase from *Gracilibacillus* A7. The bacterial strain *Gracilibacillus* A7 (GenBank accession no. EU262659) was described in our previous study.[16] For the purification of extracellular alginase, *Gracilibacillus* A7 cells were aerobically cultured at 30 °C and 150 rpm for 96 h in liquid media consisting of 5 g of polypeptone, 1 g of yeast extract, 5 g of sodium alginate, and 22.92 g of NaCl in 1000 mL of water. The pH of the medium was adjusted to 8.5. The cells were removed from the medium by centrifugation at 8000 rpm and 4 °C for 20 min. The supernatant was precipitated by 80% saturation with ammonium sulfate at 0 °C. After centrifugation at 8000 rpm and 4 °C for 20 min, the precipitated protein was collected and dissolved in 0.02 mol of phosphate−citrate buffer (pH 7.0) and used as partially purified alginase for algino-oligosaccharide production.

Analysis of Enzyme Activity. The enzyme solution (1 mL) was mixed with 1 mL of 0.5% sodium alginate solution (in a pH 7.0 phosphate buffer) in a test tube and incubated in a 40 °C water bath. After incubating for 30 min, the enzyme solution was quickly moved to a boiling water bath for 15 min to stop the reaction. Next, 3 mL of a 3,5-dinitrosalicylic acid (DNS) solution was added to the enzyme solution, boiled in a water bath for 5 min, and adjusted to a final volume of 25 mL. The solution's absorbance at 540 nm was measured to calculate the enzyme activity. A control solution, containing 1 mL of boiled enzyme solution instead of crude enzyme extract, was also measured. Glucose was used as the standard reducing sugar and to calculate the standard calibration curve according to the method described by Miller.[18] An enzyme activity unit (U) is defined as the quantity of alginase required to produce 1 μg of reducing sugar within 1 min under the experimental conditions.

$$\text{enzyme activity (U)} = C \times 25\ \text{mL} \times 1000/t \qquad (1)$$

In this equation, C is the reducing sugar content in the sample in mg/mL and t is the reaction time in min.

The protein content in the enzyme solution was determined according to the method described by Bradford[19] with the standard curve prepared using bovine serum albumin. The specific activity of the protein was expressed as U/mg.

Characterization of Optimum Conditions for Alginase Derived from *Gracilibacillus* A7. The optimum conditions for alginase were analyzed using partially purified alginase for the degradation of alginate under various conditions of (1) temperature, (2) pH, and (3) substrate concentration and (4) in the presence of various metal ions. (1) The incubation temperature was set at 20, 25, 30, 35, 40, 45, and 50 °C. To determine the optimum temperature, 1 mL of 0.5% sodium alginate (w/v) in 0.02 mol/L phosphate−citrate buffer at various temperature values was mixed with 1 mL of alginase, and enzyme activity was measured using the DNS method. (2) The incubation pH of the phosphate buffer was set at 6.0, 7.0, 8.0, 9.0, and 10.0. To determine the optimum pH, 1 mL of 0.5% sodium alginate (w/v) in 0.02 mol/L phosphate−citrate buffer at various pH values was mixed with 1 mL of alginase and incubated at 30 °C. (3) The substrate concentration of sodium alginate (mass fraction) was set at 0.1, 0.3, 0.5, and 0.7%. To determine the alginase activity, 1 mL of sodium alginate in 0.02 mol/L phosphate−citrate buffer was mixed with 1 mL of alginase. (4) Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, and Na^{+} solutions were prepared at a concentration of 50 mmol/L. The metal ion solution (0.5 mL) was added to 0.5 mL of the enzyme solution and incubated at 40 °C for 30 min after the addition of 1 mL of 0.5% alginate solution. Next, the enzyme activity was determined using the DNS method. A control solution was prepared using an enzyme solution with no metal ions.

Obtaining Oligosaccharides with Various DP Values. Oligosaccharides were produced by the partially purified enzyme using sodium alginate as the substrate. The partially purified alginase (10 mL) was mixed with 200 mL of 0.5% (w/v) alginate in a 0.02 mol/L phosphate−citrate buffer solution and incubated at 30 °C for 300 min. Samples were taken at intervals of 30, 60, 120, 180, 240, and 300 min, defined as P1, P2, P3, P4, P5, and P6, respectively, and were incubated in a boiling water bath for 15 min to stop the enzymatic reaction. After centrifugation at 8000 rpm for 15 min, the supernatant was used to determine the reducing sugars by the DNS method and to detect unsaturated sugar content by measuring the absorbance at 235 nm.[5] The total sugar content was determined by the phenol−sulfuric acid method as described by Doubois et al.[20] The average DP of the alginate fragments was calculated by dividing the reducing sugar by the total sugar according to the method described by An et al.[6]

Root Growth Promoting Experiment. The effect of oligosaccharides on the root elongation of *Brassica campestris* L. was determined using the method described by Xu et al.[7] Briefly, 5 mL of alginase products P1−P6, which were collected after various times throughout the reaction, were poured onto a filter paper at the bottom of a 10 × 100 cm Petri dish. Twenty seeds were placed in the dish and incubated in the dark. The experiment was conducted in triplicate with a 0.02 mol/L phosphate−citrate buffer solution as the control. The root length was measured after 3 days, and the relative root length (RRL) was calculated by dividing the root length of the control by the lengths of the roots exposed to the extract and multiplying by 100. The effect of salt stress on the process of root elongation was examined by adding NaCl to the plant cultivation system to a final concentration of 85 mM.

Analysis of the Antioxidative Enzymes in Plant Roots. Plant roots were cut, and 0.5 g of fresh tissue was homogenized in an ice-cold mortar. Sodium phosphate buffer containing 1 mM sodium ascorbate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 M NaCl at pH 7.0 was used at a concentration of 1:10 (w/v) in the mortar. After centrifugation at 10000*g* for 20 min, the supernatant was used for further measurements.

The SOD activity was assayed by the photochemical nitroblue tetrazolium (NBT) method.[21] The reaction mixture (2 mL) contained 35 mM phosphate buffer (pH 7.8), 4 μM riboflavin, 13 mM methionine, 0.1 μM EDTA, 75 μM NBT, and 100 μL of protein extract. A reaction mixture containing no protein extract was used as the control. One unit of SOD was defined as the quantity of enzyme necessary to inhibit the NBT reaction by 50% per minute.

The POD activity was measured according to the method of Gajewska and Skłodowska.[12] The assay mixture contained 50 mM sodium acetate buffer with 5.4 mM guaiacol, 15 mM H_2O_2, and enzyme extract at pH 5.6. The change in absorbance due to the oxidation of guaiacol to tetraguaiacol (e = 26.6/mM/cm) was detected at 470 nm while simultaneously assaying reference samples lacking H_2O_2. Enzyme activity was expressed in units, which represent 1 μmol of tetraguaiacol formed per minute.

CAT activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm, as described by Dhindsa et al.[22] The changes in absorbance at 240 nm were read every 10 s for 90 s using a spectrophotometer. The enzyme activity was calculated using a molar extinction coefficient for H_2O_2 of 36 M/cm, and the results were expressed in μmol/min/gFW (fresh weight).

The level of lipid peroxidation was determined by measuring the thiobarbituric acid reactive substance (TBARS) levels following the method of Shalata and Tal.[23] The samples were mixed with 1 mL of 10% TCA and 1 mL of 0.67% thiobarbituric acid (TBA) and heated in a boiling water bath for 15 min. The TBARS level was determined by detecting the absorbance at 535 nm and was expressed as μM MDA/gFW.

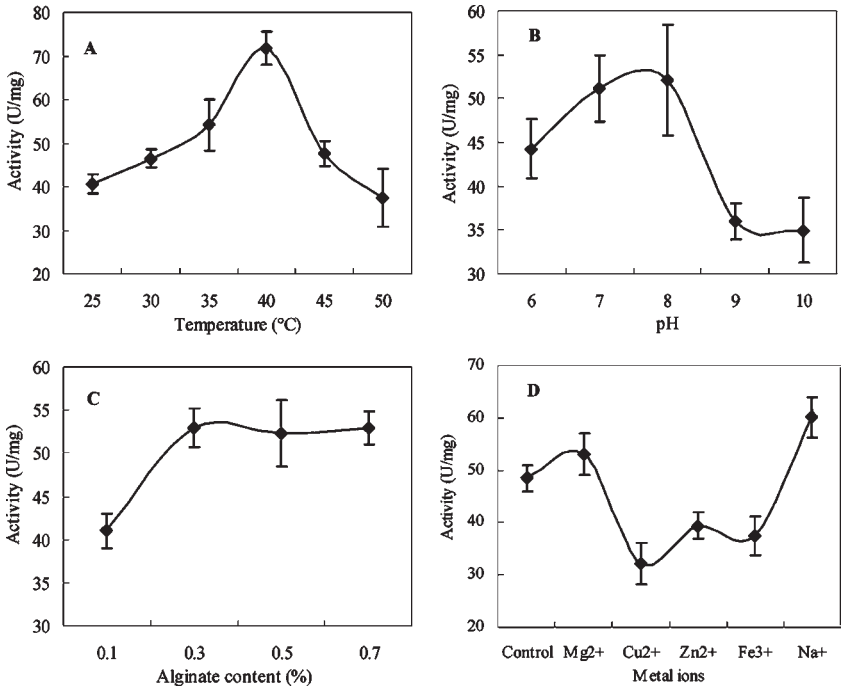

Figure 1. Characterization of the activity of alginase from *Gracilibacillus* A7 under various conditions of (A) temperature, (B) pH, (C) alginate content, and (D) metal ions. The experiment was conducted using partially purified alginase in a reaction system with 0.5% alginate and a pH 7.0 phosphate buffer solution. Values represent the mean ± SE (n = 3).

Statistical Analysis. All indices were determined from measurements of three independent experiments. Data were subjected to analysis of variance (ANOVA). Sample variability was estimated by the standard deviation of the mean. The statistical differences between treatments with and without the addition of salt in the root elongation experiment were determined using the Student's paired t test. The differences were considered to be significant when the probability was <0.05.

RESULTS AND DISCUSSION

Characterization of Alginase from *Gracilibacillus* A7. Changes in the activity of alginase derived from *Gracilibacillus* A7 under various temperatures, pH conditions, and substrate concentrations and in presence of metal ions are depicted in Figure 1. The optimum temperature for *Gracilibacillus* A7 alginase is 40 °C, yielding an activity of 71.6 U/mg. The enzyme activity was lower at temperatures higher or lower than 40 °C. Similar enzyme activity was observed at temperatures under 25 °C and above 50 °C, suggesting that the enzyme activity was inhibited by both high and low reaction temperatures. The enzyme activity increased from a pH of 6 to 8, and the maximum activity of 52.1 U/mg was obtained at a pH of 8. Furthermore, the enzyme activity decreased to approximately 35 U/mg at higher pH values of 9 and 10. The enzyme activity was stable when alginate concentrations ranged from 0.3 to 0.7%, but decreased when the alginate concentration was lower than 0.1%. This result suggests that a minimal level of alginate content is required to maintain a high level of alginase activity. Among the various metal ions tested in Figure 1D, Na^+ and Mg^{2+} increased enzyme activity by 23.4 and 9.6% compared with the control, respectively, whereas Cu^{2+}, Zn^{2+}, and Fe^{3+} inhibited enzyme activity.

The effect of temperature on alginase activity was also analyzed by An et al.[6] and Shimokawa et al.[24] As *Gracilibacillus* A7 was isolated from compost, the optimum temperature for *Gracilibacillus* A7 alginase was higher than that of several other strains, such as *Bacillus* sp. ATB-1015[25] and *Pseudomonas* sp. N7151-6.[26] Although the optimum pH is alkaline, the activity of the enzyme decreased sharply at high pH levels, suggesting that a lower pH may be favorable for the *Gracilibacillus* A7 alginase. Alginate lyases exhibit a wide range of optimum pH values, occasionally as low as pH 5.[25] Some alginases generally depend on Mn^{2+}, Mg^{2+}, Ni^{2+}, and Ca^{2+} for optimal activity.[1] At a concentration of 2 mM, Fe^{3+} reduced the activity of alginase from *Streptomyces* sp. A5 by 96%, and Cu^{2+} also displayed an inhibitory effect.[11] However, Cu^{2+} and Zn^{2+} stimulate the activity of alginase in some cases.[27] The optimum conditions for various types of alginases may differ greatly depending on the source of the alginate-degrading microorganisms.

Changes in Reducing Sugars, Unsaturated Sugars, and DP Values during the Degradation of Alginate by *Gracilibacillus* A7-Derived Alginase. Figure 2 indicates changes in reducing sugars and unsaturated sugars during the degradation of alginate by the alginase derived from *Gracilibacillus* A7. During the reaction time, the level of reducing sugars initially increased rapidly within the first 60 min. In contrast, an initially slow increase in the level of unsaturated sugars was observed, followed by a rapid increase after the first 60 min. This result indicates that two kinds of enzymes may be included in the alginase, which divide the degradation process into two phases.[2] The first phase is characterized by a rapid increase in reducing substances that is mediated by a currently uncharacterized hydrolytic activity of the alginase enzyme complex; the second phase is characterized by a significant decrease in reducing substances and a rapid increase in unsaturated monouronic and 4-deoxy-5-ketouronic acid. In some cases, certain enzymes degrade the alginate polymer in an exolytic manner, which decreases the viscosity and increases the level of reducing sugars at a slow rate.[4]

The changes in the DP values at various time points during the reaction are presented in Figure 3. The DP value decreased by nearly half, from 87 to 49, during the initial 60 min. In one study, this decrease in the DP value was found to be accompanied by a reduction of viscosity.[6] After 240 and 300 min, the DP value decreased to 5.4 and 3.3, respectively. This result clearly indicates that the *Gracilibacillus* A7 enzyme has endoalginate lyase characteristics and demonstrates that low molecular weight oligosaccharides were produced over an extended reaction period. Three major unsaturated oligouronides with DP values of 7−8, 5−6, and 3−4 were detected at concentrations close to 100% as end-products of the polyM block, polyG block, MG random block, and Alg-Na after 12 h of reaction time.[28]

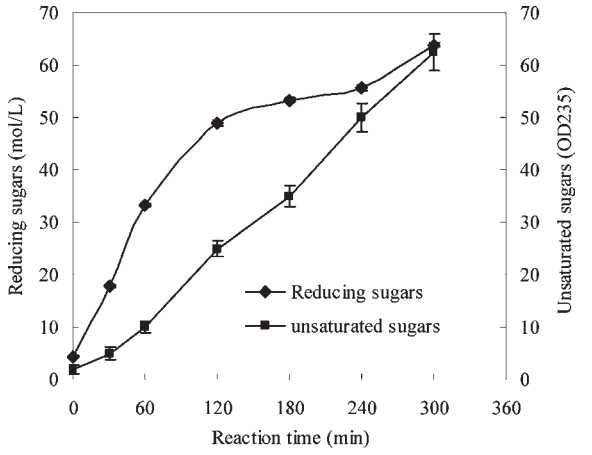

Figure 2. Changes in reducing sugars and unsaturated sugars at various time points by *Gracilibacillus* A7 alginase. Reducing sugars were analyzed by the DNS method and expressed as glucose concentration (mol/L), and unsaturated sugar content was determined by measuring the absorbance at 235 nm. Values represent the mean ± SE (n = 3).

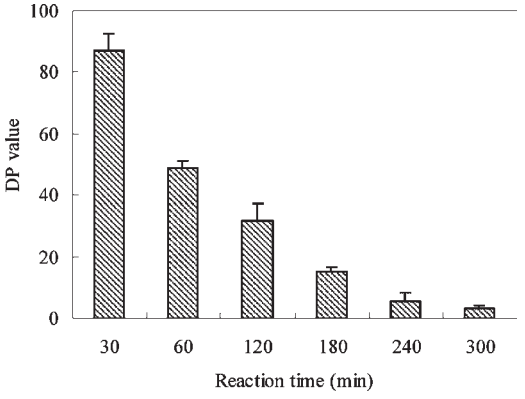

Figure 3. Degree of polymerization (DP) generated by *Gracilibacillus* A7 alginase at various time points. The DP value was obtained by dividing the reducing sugar into the total sugar. Values represent the mean ± SE (n = 3).

Elicitor-Active Effect of Oligosaccharides on Root Growth and Antioxidative Enzyme Activity Analysis. In Figure 4, the effects of various oligosaccharides on the process of root elongation are characterized and compared with those observed under salt stress with 85 mM NaCl. In the control experiment without the addition of oligosaccharides, the RRL value decreased to 64% after the addition of salt. The RRL values increased as the DP values decreased, reaching maximum values of 135% without salt in P6 and 118% with salt in P5. Significant differences (P < 0.05) were observed between treatments with salt and without salt in all of the oligosaccharides except P5. However, the differences of RRL values between no salt and salt stress decreased with reduced DP values, suggesting that the oligosaccharides relieved the salt stress during the plant growth process. A negative correlation was also observed between the RRL values and the DP values, both with no salt and under salt stress (Figure 4B). The low molecular weight oligosaccharides were more effective in enhancing plant growth and root elongation, which was also demonstrated by other researchers.[6] The dependence of root growth promoting activity on the DP of the uronates was tested, and the pentamer was determined to have the highest activity.[7]

Figure 5 depicts changes in antioxidative enzymes (SOD, POD, and CAT) and lipid peroxidation (as measured by MDA) in plant roots in the presence of oligosaccharides with various DP values and under salt stress. Elevated enzyme activity was observed under salt stress compared to the absence of salt for SOD and POD activities and MDA value, although CAT activity was reduced by treatment with salt stress. In the absence of salt stress, the SOD activity increased with decreasing DP values. Under salt stress, the SOD activity decreased after P3 and remained stable under lower DP values. The POD value decreased with reduced DP values, both under salt stress and without salt stress. However, the differences in the enzyme activities between no salt and salt treatment became smaller with decreased DP values after

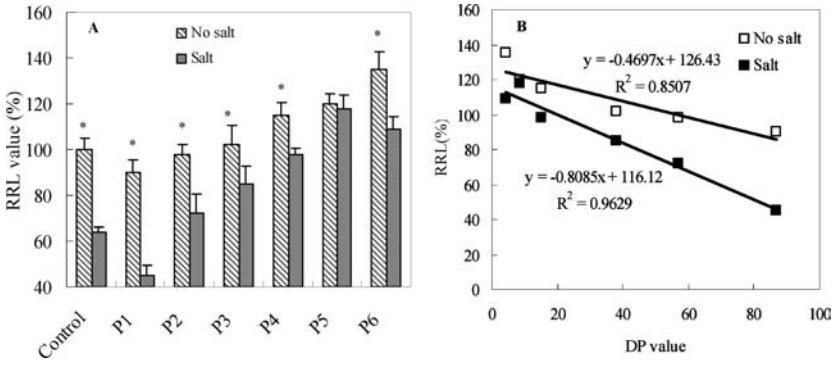

Figure 4. Relative root length (RRL) values during incubation with oligosaccharides with various degree of polymerization (DP) values. "No salt" indicates that no extra NaCl was added to the incubation system; "salt" indicates that 85 mM NaCl was used in the experiment to test salt stress. RRL values in P1−P6 are relative to the buffer solution control value. ∗, P < 0.05 indicates a significant difference for salt-stress treatment versus no salt treatment for the oligosaccharides with different DP values by ANOVA test.

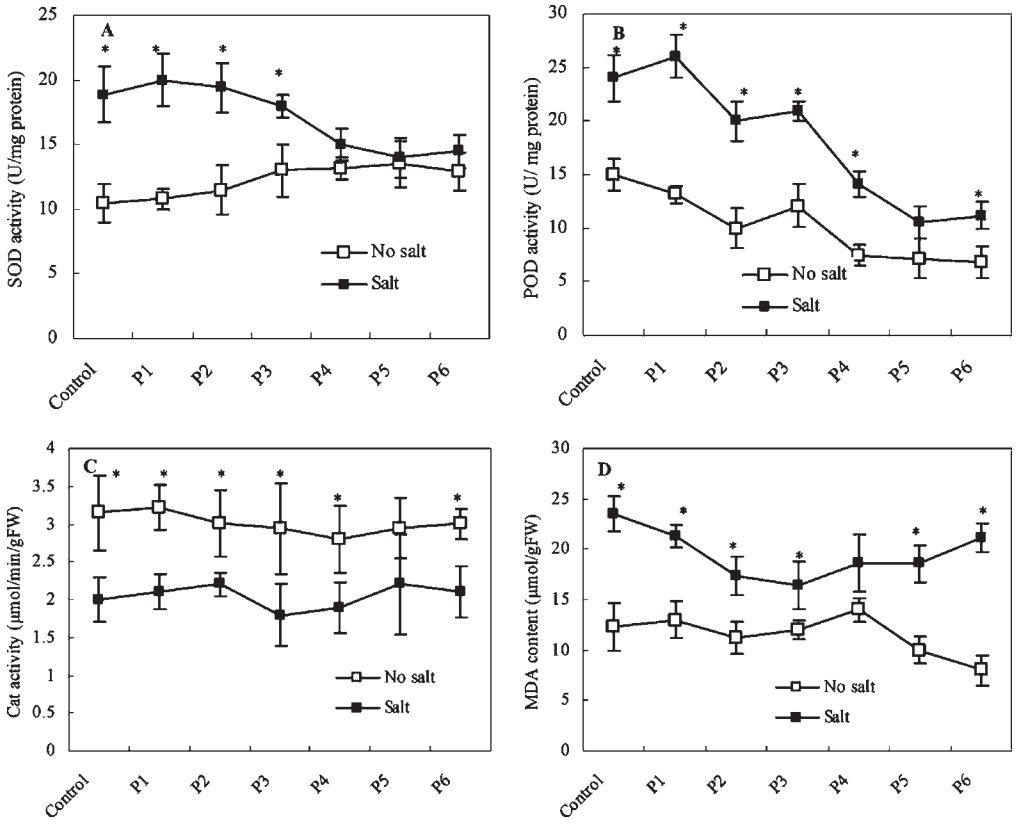

Figure 5. Change in antioxidative enzyme activity and malondialdehyde (MDA) values in the roots of plants during incubation with oligosaccharides with various degree of polymerization (DP) values: (A) SOD activity; (B) POD activity; (C) CAT activity; (D) MDA content. "No salt" indicates that no extra NaCl was added to the incubation system; "salt" indicates that 85 mM NaCl was used in the experiment to test salt stress. Values represent the mean ± SE ($n = 3$). *, $P < 0.05$ indicates a significant difference for salt-stress treatment versus no salt treatment for oligosaccharides with different DP values by ANOVA test.

P3 in both SOD and POD detection result, which suggests that the salt-induced SOD and POD activities are alleviated by oligosaccharides with low molecular weights. Figure 5C indicates that CAT activity was reduced under salt stress but was not affected by the addition of oligosaccharides. The MDA content was elevated under salt stress; however, the MDA content decreased at lower DP values in the treatments without salt stress. Under salt stress, the MDA value was lower when the DP values were low (15−32 for P3 and P4), suggesting that a moderate molecular mass is favorable for relieving the effects of salt stress on lipid peroxidation activity.

Although the effect of oligosaccharides on the process of root elongation has been widely observed, the antioxidative enzyme activity of plants growing under various molecular masses of oligosaccharides and salt stress has not been characterized. Our research indicates that the oxidative stress induced by elevated salt content can be relieved by the oligosaccharides produced from alginate degradation. The distinguishing characteristics of the alginase are the key factors in determining the properties and DP values of the degradation products. The effects of the oligosaccharides on the antioxidative enzyme may be attributed to the following: (1) oligosaccharides act as osmoprotective agents[29] and (2) oligosaccharides may also demonstrate an auxin-like mode of action such as indole-3-butyric acid (IBA). Some oligosaccharides, such as galactoglucomannan oligosaccharides (GGMOs), may interact with IBA and inhibit the function of low concentrations of IBA.[30] Considering the higher reducing sugar content depicted in Figure 2, the osmoprotective effect may be the main mechanism by which alginate oligosaccharides relieve salt stress for *B. campestris* L. in this study.

In this study, the alginase from *Gracilibacillus* A7 was characterized under the optimum reaction condition of 40 °C at an alkaline pH of 8; the activity of *Gracilibacillus* A7 alginase was higher at an alginate content of over 0.3% and can be stimulated by Na^+ and Mg^{2+} metal ions. The levels of reducing sugars and unsaturated sugars increased with the length of the *Gracilibacillus* A7 alginase reaction time, and a preferentially rapid increase in reducing sugars was observed at the initial stage of the reaction. The molecular mass of the oligosaccharides decreased with the reaction time, as indicated by the DP values. The plant growth experiment suggests that low molecular weight oligosaccharides were more effective in stimulating root elongation and relieving salt stress. The antioxidative enzymes in the plant roots behaved differently under the addition of oligosaccharides with various DP values and under salt stress. Salt stress induced higher levels of SOD, POD, and MDA activity, which can be reduced by the elicitor-like effect of the oligosaccharides. These results suggest that oligosaccharides derived from the degradation of alginate may be applied in agriculture to deal with the inhibition of plant growth in saline and alkaline soils.

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