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Antibacterial Mechanism of Ethyl Acetate Extracts From Naked Oat Against *Bacillus subtilis*

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Abstract - The antibacterial mechanism of the ethyl acetate extracts from naked oat against *Bacillus subtilis* were studied in this paper based on assays such as cell wall alkaline phosphatase (AKP), permeability and integrity of cell membrane as well as electron microscopy observations. The results showed that the ethyl acetate extracts had significant effects on AKP, permeability and integrity of cell membrane. We concluded that the mechanism of action of the ethyl acetate extracts against *B. subtilis* might be described as:Acting on cell wall and membrane, resulting in disruption, cell lysis, and the leakage of intracellular constituents according to the results of AKP, the leakage of electrolytes, the losses of contents (proteins, reducing sugars and 260 nm absorbing materials) assays and electron microscopy observations. Overall, the results clearly indicated that the ethyl acetate extracts from naked oat was potential to control the contamination of foods caused by bacterial diseases.
Keywords: Naked oat; Antibacterial mechanism; Extracts; *Bacillus subtilis*

Introduction

In recent years, food poisoning and food spoilage caused by microorganisms are the most important issues, and there has been a dramatic increase throughout the world in respect to the number of reported cases for food-borne illness (Sokmen *et al.*, 2004; Shan *et al.*, 2007). For many years, a variety of chemicals and synthetic compounds have been made to control microbial growth. However, consumers have grown concerned about the side effects of synthetic chemicals and want safer materials for preventing and controlling oxidation and pathogenic microorganisms in foods (Alzoreky & Nakahara, 2003). Plants can be an excellent source of natural antibacterial agents and can be effectively used in the food industry to preserve the quality and improve the shelf-life of food products (Tiwari *et al.*, 2009). In addition, as in most of the cases, plants or their extracts are believed to be safe, and non-toxic to humans (Burt 2004; Rymbai *et al.*, 2011).

Oat, a cereal for human or animal consumption, has received increased interest because of its excellent health-related properties, such as high contents of solube dietary fibre and well-balanced protein, energy in the form of carbohydrate and oil, and several vitamins and minerals (Petkov *et al.*, 2001). In addition, oats contained abundant antioxidant compounds such as tocols (Emmons *et al.*, 1999), phytic acid (Miller *et al.*, 1980), sterols (Moreau *et al.*, 1996), and phenolic compounds (Peterson, 2001). Phenolic compounds exhibited a wide range and different biological effects such as antioxidant, anti-inflammatory, antiallergic, anti-carcinogenic activities (Peterson *et al.*, 2002; Chen *et al.*, 2004; Sur *et al.*, 2008), and antimicrobial activity from extracts of the oats (Shin *et al.*, 2005; Bahraminejad *et al.*, 2008; Sørensen *et al.*, 2010). In previous study, we had investigated the antibacterial properties of different consecutive extracts from naked oats (*Avena*

nuda L.) on the growth of food-related bacteria, and the ethyl acetate extracts from oats had been found to be the highest antibacterial activities against the tested bacteria. Particularly, *B. subtilis* had shown the most sensitive activity to essential oil by showing the lowest MIC and MBC values of 31.2 μg/mL (Hao *et al.*, 2014**)**. However, to the best of our knowledge, little work has been reported on the mechanism of action of extracts from naked oats on the growth of microorganisms. Therefore, the objective of this work was to investigate the mechanism of action of the ethyl acetate extracts oats against *B. subtilis*, which would provide some foundational information for the developing and application of oats.

Materials and Methods

Plant materials and chemicals

A naked oat cultivars (*Avena nuda* L.), Bayou I, were used in the study. The cultivars were all grown in 2012 in bases for growing organic oat, Shanxi, China, and they are the main commercial cultivars in local area. The harvested oat grains were dried to about 12% moisture. Nutrient agar (NA), nutrient broth (NB) and tryptone soy agar mediums were from Beijing Aoboxing Bio-tech Co. Ltd. (Beijing, China). Other chemicals used were all of analytical grade. The *Bacillus subtilis* ATCC 6051 are provided by the College of Life Science, Shanxi Normal University, and cultured at 37 °C on NA or NB mediums.

Preparation of extracts

The dried oats were finely ground with a micro plant grinding machine (FZ102; Tianjin Taisite Instruments, Tianjin, China), and the powder was sifted by 60-meshes. The powder (50 g) was extracted with 500 mL ethyl acetate and shaken with a laboratory rotary shaker at 150 rpm for 8 h at 30 °C, and then the homogenates were centrifuged for 10 min at 4 °C and 5,000 g in a centrifuge (Eppendorf 5417R, Germany). The homogenates were evaporated and dried under vacuum (below 40 °C), to yield the ethyl acetate extracts.

Cell wall alkaline phosphatase (AKP)

The AKP was determined according to the method described by Ali *et al.* (2015) with some modifications. After incubated at 37 °C for 10 h, the extracts at three different concentrations (control, MIC and 2×MIC) were added to the medium and then the culture medium was centrifuged at 4,000 rpm for 5 min at selected time intervals. The AKP of the supernatant was determined using a StemTAG™ Alkaline Phosphatase activity assay kit at various times.

Cell membrane permeability

The permeability of bacteria membrane was expressed in the relative electric conductivity and determined according to the method described by Kong *et al.* (2008). After incubated at 37 °C for 10 h, *B. subtilis* strains were separated by centrifugation at 5000 rpm for 10min. Then the bacteria were washed with 5% of glucose until their electric conductivities were near to that of 5% glucose. The extracts at three different concentrations (control, MIC and 2×MIC) were added to 5% glucose and the electric conductivities of the mixtures were marked as L_1. Then different concentrations of extracts were added into the isotonic bacteria solution. After completely mixed, the samples were incubated at 37 °C for 8 h, and then the conductivities were measured and marked as L_2. The conductivity of bacteria in 5% glucose treated in boiling water for 5 min was served as the control and marked as L_0. The permeability of bacteria membrane is calculated according to the formula, the relative electric conductivity (%) $=100\times (L_2 - L_1)/L_0$.

Integrity of cell membrane

The cell integrity of *B. subtilis* strains was examined by determining the release of cell constituents into supernatant according to the method described by Du *et al.* (2012), with slight modifications. Cells from the 100 mL working-culture of tested *B. subtilis* were collected by centrifuged for 15 min at 5000 rpm, washed three times, and resuspended in 0.1 M phosphate buffer solution (PBS, pH 7.4). One hundred milliliters of cell suspension were incubated at 37 °C under agitation for 4 h in the presence of extracts at three different concentrations (control, MIC

and 2×MIC). Then, 25 mL of samples were collected and centrifuged at 11,000 g for 5 min. And then the concentrations of proteins and reducing sugars in supernatant were determined according to the method described by Xu *et al.* (2010). In addition, to determine the concentration of the released constituents consisting largely of nucleic acids, 3 mL supernatant was used to measure UV absorption at 260 nm. Correction was made for the absorption of the suspension with the same PBS containing the same concentration of essential oil after 2 min of contact with tested strains. The untreated cells were corrected with pH 7.4 PBS.

Transmission electron microscope (TEM) observation

TEM observation was performed as given by Diao *et al.* (2013). The bacteria cells were incubated for 10 h in NB at 37 °C. The suspension was added 1×MIC of the extracts, respectively; control cultures were left untreated. The suspension was incubated for 4 h at 37 °C respectively, and then the suspension was centrifuged. The cells were washed twice with 0.1 M PBS (pH 7.4) and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS overnight at 4°C. After this, **t**he cells were postfixed with 1% (w/v) OsO_4 in 0.1 M PBS for 2 h at room temperature and washed three times with 0.1 M PBS, then dehydrated by a graded series of ethanol solutions (30%, 50%, 70%, 90%, and 100%). Stained bacteria were viewed and photographed with a transmission electronic microscope (H-7650, HITACHIL Ltd., Japan) operated at 80 kV, and were analyzed with the digital imaging software.

Statistical analysis

All results are expressed as mean ± SD (n=3). The data were subjected to one-way analysis of variance (One-way ANOVA) and followed by a Duncan's multiple range test at significant level being considered at $P < 0.05$.

Results and Discussion

Cell wall alkaline phosphatase (AKP)

The Effects of the ethyl acetate extracts from oats on cell wall AKP of *B. subtilis* was evaluated at 1×MIC and 2×MIC concentrations (Figure 1a). As observed in Figure 1a, compared to the control, *B. subtilis* treated with the essential oil at the 1×MIC value showed an obvious increase in AKP contents over the first 3 h period of the test, and increased to 5.9 μg/mL. In contrast, the AKP contents did not show obvious change as time prolonging. Similar to the change trend at 1×MIC, in treatments at 2×MIC, the AKP content increased obviously to 9.2 μg/mL at the third hour, and after that no obvious change was found. The changes of increase were much higher than the control group at whole processing. It could be concluded that the ethyl acetate extracts from oats had great damaging effect on cell walls of *B. subtilis*.

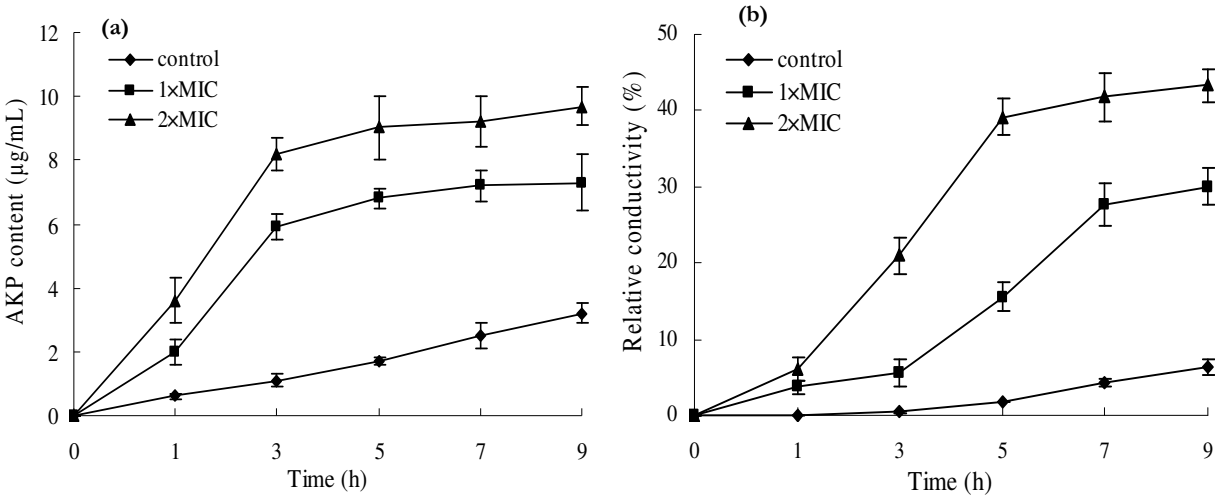

Figure 1. (a) Effect of the ethyl acetate extracts from oats on the impermeability of AKP, (b) Effect of the ethyl acetate extracts from oats on the impermeability of cell membrane

Cell membrane permeability

Further antibacterial mode of action of extracts against the tested food-borne pathogens was confirmed using the assay of cell membrane permeability. Figure 1b showed the effect of the ethyl acetate extracts from oats on the membrane permeability of *B. subtilis*. There was little change in the relative electric conductivity of the control during the first 3 h period of the test, and then an increase in the relative electric conductivity was found, which may be due to normal lysis and death of bacteria, resulting in the rise in the relative electric conductivity. Comparing to the control, the relative electric conductivity of the suspensions increased immediately after the addition of extracts at greater than or equal to MIC concentration and it also increased rapidly with the increasing treatment time and concentration of extracts. It meant that the permeability of bacteria membrane would be increased correspondingly, which caused the leakage of intracellular ingredient, especially losses of electrolytes including K^+, Ca^{2+}, Na^+ and so on.

Integrity of cell membrane

The integrity of cell membrane was determined by the measurement of the release of cell constituents including protein, reducing sugar and the absorbance at 260 nm of the supernatant of tested bacteria. Table 1 showed the results when *B. subtilis* were treated with different concentrations of ethyl acetate extracts from oats for 4 h, respectively. The results indicated that after adding the corresponding essential oil to strains, the cell constituents' release increased significantly with the increased concentration of the essential oil. Compared to control, the concentration of proteins, reducing sugars and cell constituents (OD_{260nm}) in suspensions treated with 1×MIC essential oil increased by 4.05, 2.81, 10.33 times respectively, while they increased by 6.92, 5.06, 18.78 times respectively when treatment at 2×MIC. These results indicated that the irreversible damage to the cytoplasmic membranes might occur, which led to the losses of cell constituents such as protein and some essential molecules and to cell death.

Table 1. The effects of the extracts on cell constituents' release of tested *B. subtilis*. Values represent means of three independent replicates ± SD. Different letters within a column indicate statistically significant differences between the means ($P< 0.05$).

Concentrations	Cell constituents release		
	Protein (μg/mL)	Reducing sugar (μg/mL)	Cell constituents (OD_{260nm})
Control	14.5± 1.1 c	12.4±1.2 c	0.018±0.004 c
1×MIC	73.2±5.1 b	47.3±2.4 b	0.204±0.011 b
2×MIC	114.8±17.6 a	75.1±7.7 a	0.356±0.028 a

Electron microscope observation

The *B. subtilis* were treated with the ethyl acetate extracts from oats at 1×MIC for 4 h, and then the morphological and physical changes of treated strains were observed by TEM. Figure 2 showed the TEM photographs of *B. subtilis* untreated and treated by the ethyl acetate extracts from oats. It was observed from TEM photographs that the cell morphology and sructure of the untrested bacteria remianed intact and regular. Discernible cell wall and cytoplasmic membrane were clearly observed as well as uniformly disturbed cytochylema and electron dense inside the bacterial cell (Figure 2a). However, cells treated with the ethyl acetate extracts were damaged in different degrees (Figure 2b). Some of the exposed cells turned from normal rod-shape into irregular cell morphology, and the electron dense inside the bacterial cell became uneven. The cell walls were broken and the cytoplasmic membrane were thin and even hard to distinguish which may give rise to the leaching out of cell content, which indicated that the ethyl acetate extracts

from oats may have severe effects on the cell wall and cytoplasmic membrane. Basically, it was consistent with this study and Yi *et al.* (2010) reported that tea polyphenols inhibited *Pseudomonas aeruginosa* through damage to the cell membrane, and that tea polyphenols influenced the membrane proteins which may have induced the metabolic disorder of the bacteria and resulted in their death. Si *et al.* (2006) also found that the antimicrobial activity of tea had a direct link with its specific phenolic compound.

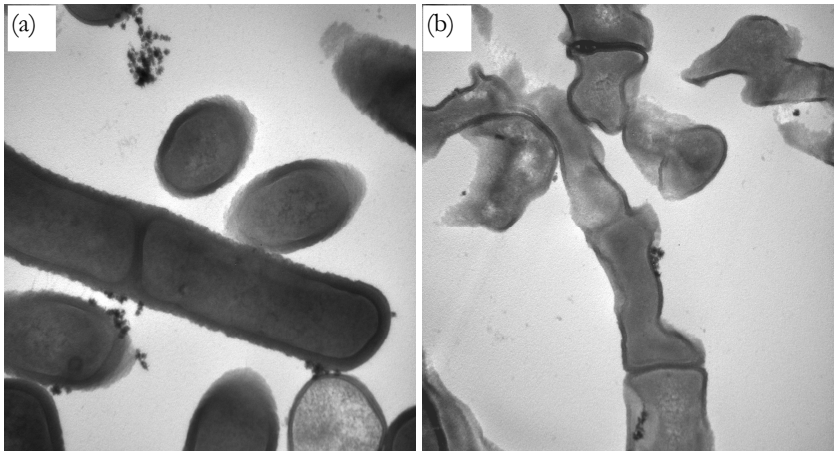

Figure 2. The photograhs of TEM of *B. subtilis* untreated (A0) and treated with the extracts at 1×MIC (A1)

Conclusions

Based on the present research, we concluded that the mode of action of the ethyl acetate extracts from oats against *B. subtilis* may be described as extracts was passing firstly through cell wall and the permeability of cell membrane was associated with generalized the integrality of membrane-disrupting effects, leading to the leakage of electrolytes as well as losses of proteins, reducing sugars, and 260 nm absorbing materials. These changes resulted in cell decomposition and death eventually. However, further research is still necessary to fully understand the mechanisms involved against other food-borne pathogens, the chemical compositions, as well as the interactions with other food ingredients in order to justify the real applications of the ethyl acetate extracts from oats in food practices as a natural antibacterial agent.

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