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Antibacterial Mechanism of Catfish Bone Hydrolysate Revealed by Atomic Force and Transmission Electron Microscopy

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Abstract. The aim of this study was to investigate the effect of the catfish bone hydrolysate (CBH) on morphology of bacteria which were observed by atomic force microscope (AFM) and transmission electron microscope (TEM). The CBH was found to inhibit *Escherichia coli* (*E. coli*) growth. The CBH at 10 mg/ml caused the significant fragmentariness in the bacterial membrane and a severe volume decrease. A possible mechanism is that CBH damages the structure of bacterial cell membrane which causes *E. coli* bacteria to die eventually.

Introduction

With the increased yield of farm-raised catfish, catfish has been processed into a variety of products. The process produces a lot of byproducts including bones, skins, viscera and heads, which account for more than half of the total fish weight[1]. Fish bones are one of the major byproducts with a wet weight accounting for 16.3% of the total wet weight of live fish and 25.0% of the dry weight [2]. Moreover, fish bones are perishable due to rich protein and fat contents[3], thus they are an important source of environmental contamination[4]. Thereby, technology development for utilizing these bones would not only provide economic benefits' but also reduce environmental stress. In our previous work, we prepared the catfish bone hydrolysate (CBH). Despite the CBH with the antibacterial property [5], we have not understood the mechanism of action. The aim of the present work was to study the antibacterial mechanism of CBH by atomic force and transmission electron microscopy.

Experimental Methods

Catfish bone hydrolysate (CBH) preparation. Channel catfish (*Ictalurus punctatus*) was kindly provided by Naruhito Aquaculture Co. (Tianjin, China). In the laboratory, the fresh fish was washed twice with tap water and then killed. The bones were mechanically separated and were rinsed with cold distilled water to clean the meat residues that adhered to the bones. After that bones were heated at 85 ºC to aid in inactivating the endogenous enzymes and to facilitate the removal of the fat. The heat- treated bones were allowed to cool and dry. The dried bones were ground to 1–3 mm particles in diameter and stored at –20 ºC in polyethylene bags until use. The dried catfish bone sample was applied to hydrolyse by food grade pepsin (Nuoao Enzyme Co., Tianjin, China). The enzymatic hydrolysis was carried out at pH 3.5, reaction temperature of 40 ºC, enzyme-substrate ratio (E/S) of 1.97 g/100 g, substrate concentration (S) of 15 g/100 mL and reaction time of 4 h. After that, the mixture was heated in a boiling water bath for 10 min in order to inactivate the enzyme, rapidly cooled to ambient temperature in an ice bath and centrifugated at 2218 g for 20 min. The supernatants were lyophilized and stored at –20 ºC until use.

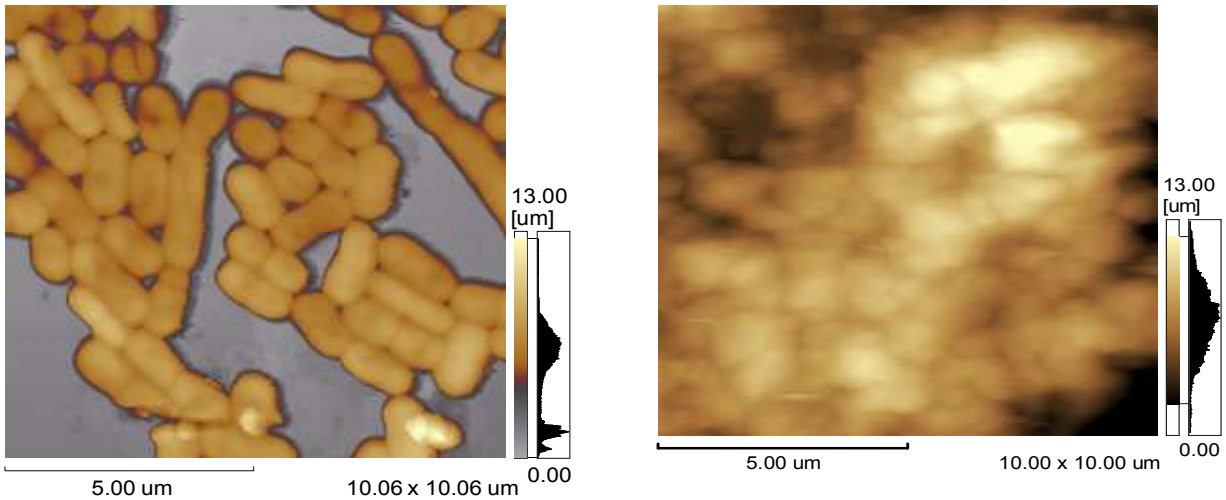
Bacteria. *Escherichia coli* strain TAU8739, stored by Tianjin Agricultural University, China, was grown overnight in LB (Luria-Bertani) broth at 37 °C. The bacteria were centrifuged and washed twice using cold phosphate buffered saline (PBS) (pH 7.2, 0.1 M) and resuspended in cold PBS (pH 7.2, 0.1 M) with or without the addition of CBH. The bacterial concentration was adjusted to 10^6 CFU/ml and the concentrations of CBH with 10 mg/ml.

Atomic force microscopy (AFM). After incubation 6 h at 37 °C, the bacterial suspensions were centrifuged and washed twice using deionized distilled water. Bacteria fixation was done in 2.5% glutaraldehyde in phosphate buffer (pH 7.2, 0.1 M) overnight and then rinsed 3 times for 10 min using cold sterile deionized distilled water. The samples were resuspended in 100 μl cold sterile deionized distilled water. The samples of 5 μl were applied on a freshly cleaved mica surface and air-dried. A scanning probe microscope (SPM-9700, Shimadzu, Kyoto, Japan) were used to image both the controls and CBH treated bacteria in air using the tapping mode with 1.5 Hz scan rate.

Transmission electron microscopy (TEM). After incubation 0.5 h and 6 h at 37 °C respectively, the bacterial suspensions were centrifuged and washed twice using deionized distilled water. Bacteria fixation was done in 2.5% glutaraldehyde in phosphate buffer (pH 7.2, 0.1 M) overnight followed by a post-fixation in 1% osmium tetroxide for 2h. Samples were then rinsed 3 times for 10 min in phosphate buffer (pH 7.2, 0.1 M). Dehydration was performed in a graded alcohol series (30, 50, 70, 80, 90, 95, and 100% ethanol). Dehydrated samples were embeded in Epon 812, sectioned at a thickness of 50 nm with a diamond knife on a Leica Ultracut equipped with a cryo-attachment (Leica, Wetzlar, German) and staining with uranyl acetate followed by Reynolds' lead citrate. The ultrathin sections were viewed and photographed using a transmission electron microscope (HITACHI-7500, Tokyo, Japan).

Results and Discussion

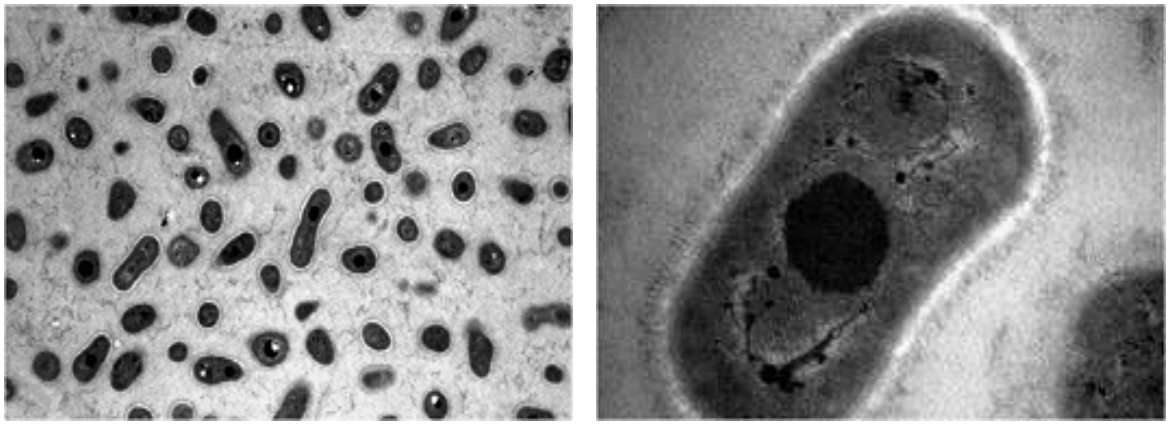
AFM images of *E. coli* treated with or without the CBH. The AFM images of *E. coli* were shown in Fig.1. The AFM image of *E. coli* untreated with CBH was shown in Fig.1 (a) and the AFM image of *E. coli* treated with CBH at the concentration of 10 mg/ml for 6 h was shown in Fig.1 (b). Fig. 1 presents the bacterial adhesion to mica was successful, in agreement with previous report[6]. Fig. 1 (a) shows a typical untreated *E. coli* cells. Image of untreated *E. coli* cells revealed characteristic rod shape and a relatively smooth surface with no ruptures or large pores similar to that observed by Li et al.[7]. AFM image of *E. coli* treated with CBH at the concentration of 10 mg/ml for 6 h is presented in Fig. 1 (b). The CBH caused the surface corrugation of the bacteria. Their morphologies were distinctly different from those untreated by CBH. It was explained that the antibacterial target of action of the CBH was damaging the cell wall.



(a) (b)

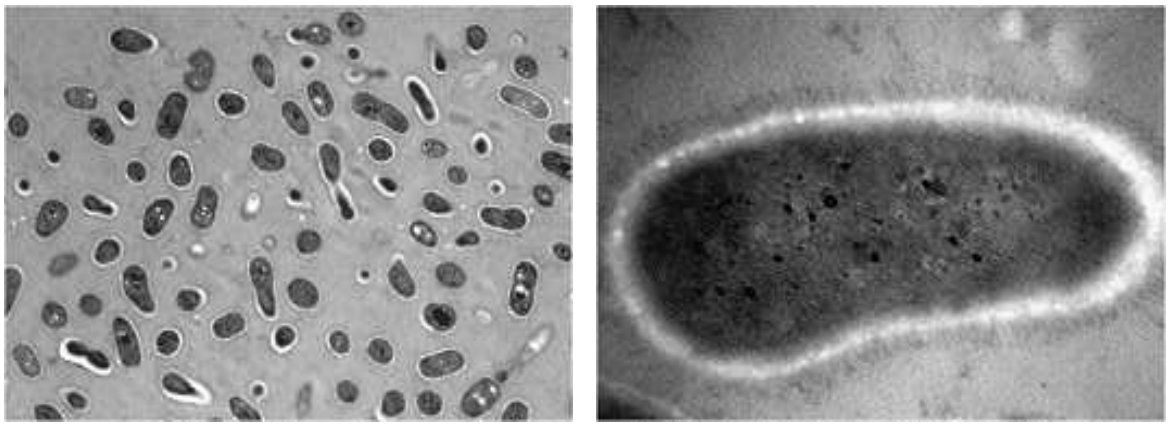
Fig. 1 Atomic force microscopy images of *Escherichia coli* (*E.coli*) treated with 10 mg/ml catfish bone hydrolysate (CBH)
(a) untreated *E.coli*; (b) treated *E.coli* with 6 h

TEM images of *E. coli* treated with or without the CBH. Though the surface morphologies of the bacteria were observed, the inner structural changes in the bacterial cells were not found using the



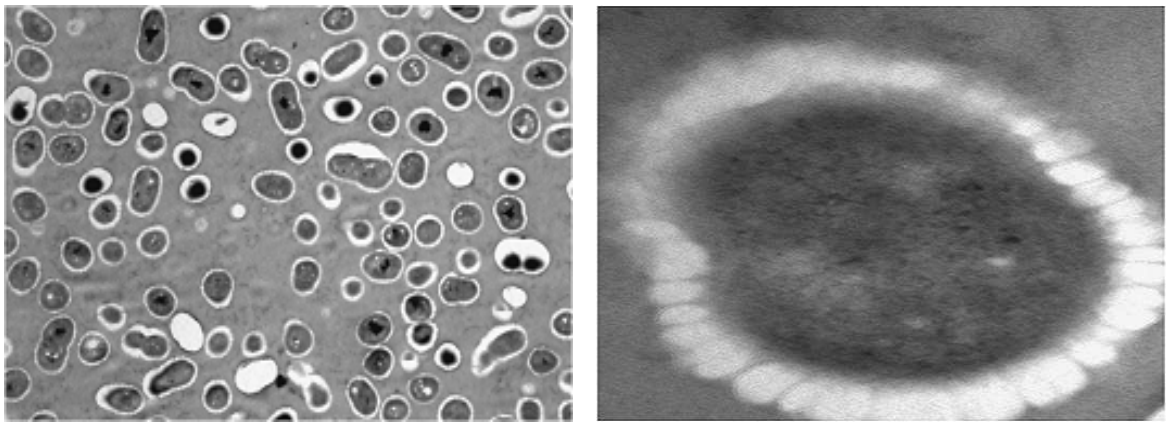
(a) (b)

Fig. 2 Transmission electron microscopy of images of *E.coli* untreated with catfish bone hydrolysate (CBH)
(a) *E.coli* (×10000); (b) *E.coli* (×100000)



(a) (b)

Fig. 3 Transmission electron microscopy of images of *E.coli* treated with 10 mg/ml catfish bone hydrolysate (CBH) with 0.5h
(a) *E.coli* (×10000); (b) *E.coli* (×100000)



(a) (b)

Fig. 4 Transmission electron microscopy of images of *E.coli* treated with 10 mg/ml catfish bone hydrolysate (CBH) with 6h
(a) *E.coli* (×10000); (b) *E.coli* (×100000)

AFM. In order to discover the in-depth antibacterial mechanism of the CBH, the *E. coli* cells treated and untreated with CBH were observed by TEM. The TEM micrographs of the *E. coli* untreated, treated with 10 mg/ml CBH for 0.5 h and treated with 10 mg/ml CBH for 6 h are shown in Fig. 2, Fig. 3 and Fig. 4 respectively. The untreated *E. coli* colony show complete profiles without visibly morphological changes in Fig. 2a. The smooth surface, integral cell wall and cell membrane, distinguishable cytoplasm structure and obvious central nuclear area of the untreated *E. coli* also are observed in Fig. 2b. The *E. coli* cells treated with CBH for 0.5 h represent slight changes compared with untreated *E. coli* cells (Fig. 3a). The CBH treated for 0.5 h causes the cell wall to shrink slightly, the cell membrane to damage, some of cytoplasm materials to reduce and the central nuclear area to disappear (Fig. 3b). The *E. coli* cells treated with CBH for 6 h represent remarkable changes of *E. coli* cells between untreated and treated with CBH for 0.5 h (Fig. 4a). The CBH treated for 6 h made the cell surfaces containing much unstained material in the periplasmic space and on the external face of the outer membranes. It resulted in the cell wall to shrink completely, the cell membrane to disappear, cytoplasm materials to reduce further, one apical end of the cell to form pore and the cell structure to collapse eventually.

The incubation time with CBH correlated well with cellular morphological changes. In general, the damage of the cell membrane increased with the increase in incubation time, with CBH having the most pronounced effect on the cell membrane damage. Some cells might have remained intact at the short incubation time. More incubation time leads to even greater cell damage of the *E. coli* cell.

Conclusions

Based on the micrograph AFM and TEM analysis, the damage pattern of *E. coli* by CBH may be described as CBH inducing damage of membrane, making a break through the permeability of membrane, resulting in the leakage of cellular materials and resulting in cell death eventually.

References

[1] H. Yang, Y. Wang, M. Jiang, J. H. Oh, J. Herring and P. Zhou: J. Food Sci. Vol. 72 (2007), p. 188-195

[2] J. N. Cameron: J. Exp. Biol. Vol. 117 (1985), p. 307-318

[3] V. Ferraro, I. B. Cruz, R. F. Jorge, F. X. Malcata, M. E. Pintado and P. M. L. Castro: Food Res. Int. Vol. 43 (2010), p. 2221-2233

[4] N. Bhaskar, T. Benila, C. Radha and R.G. Lalitha: Bioresour. Technol. Vol. 99 (2008), p. 335-343

[5] X. Ren, L. Ma, J. Chu, Y. Wang, Y. Zhuang, S. Zhang, H. Yang and H. An. J. Aquat. Food Prod. T., Vol. 21 (2012), p. 99-110

[6] M. Meincken, D. L. Holroyd and M. Rautenbach: Antimicrob. Agents Chemother. Vol. 49 (2005), p.4085–4092

[7] A. Li, P.Y. Lee, B. Ho, J.L. Ding, C.T. Lim: Biochim. Biophys. Acta. Vol.1768 (2007), p. 411 – 418

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