Antimicrobial action of achacin is mediated by L-amino acid oxidase activity

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Abstract Achacin is an antibacterial glycoprotein purified from the mucus of the giant snail, *Achatina fulica* Férussac, as a humoral defense factor. We showed that achacin has L-amino acid oxidase activity and can generate cytotoxic H_2O_2; however, the concentration of H_2O_2 was not sufficient to kill bacteria. The antibacterial activity of achacin was inhibited by various H_2O_2 scavengers. Immunochemical analysis revealed that achacin was preferentially bound to growth-phase bacteria, accounting for the important role in growth-phase-dependent antibacterial activity of achacin. Achacin may act as an important defense molecule against invading bacteria.

Key words: Achacin; Antibacterial activity; L-Amino acid oxidase; Hydrogen peroxide; Host defense; Bacteria recognition

1. Introduction

We previously isolated [1] and cloned [2] an antibacterial glycoprotein called achacin from the body surface mucus of the giant snail, *Achatina fulica* Férussac. Achacin shows strong bactericidal activity against both Gram-positive and -negative bacteria [3], and it appears to attack the plasma membranes of bacteria [4]. Achacin is thought to contribute to the innate immunity of the snail, but the mechanism by which it does so has not been elucidated.

Recent sequence analysis revealed that achacin belongs to the family of amine oxidases, which are flavin enzymes. This family comprises various amine oxidases including L-amino acid oxidase (LAO, EC 1.4.3.2). LAOs catalyze oxidative deamination of L-amino acids to produce the corresponding α-ketoacids, hydrogen peroxide (H_2O_2), and ammonia (NH_3). Some LAOs have antibacterial properties [5], thus we hypothesized that antibacterial activity of achacin is related to the H_2O_2 generated through the oxidative deamination reaction. Interestingly, snake LAOs cause induction of apoptosis [6–8] and platelet aggregation [9,10] and have antibacterial activity [5]. These effects are thought to be due to H_2O_2 produced by the enzyme.

Although LAOs exist widely among living organisms from invertebrates to vertebrates, their functions in vivo are not well understood. Here, we show that the H_2O_2-generating LAO activity of achacin contributed to its antibacterial action. Furthermore, we report that achacin specifically recognized growth-phase bacteria.

2. Materials and methods

2.1. Materials

Anti-rabbit IgG antiserum conjugated to horseradish peroxidase was purchased from Dako Japan (Kyoto, Japan). Xylenol orange was purchased from Chroma-Gesellschaft Schmid (Köngen, Germany). All other chemicals were purchased from Wako Pure Chemicals (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma (St. Louis, MO, USA).

2.2. Bacterial strains and media

Staphylococcus aureus (IAM1011) and *Escherichia coli* (K12 W3110) were used in this study. These strains were grown in heart infusion bouillon medium (Eiken Chemical, Tokyo, Japan) and Luria–Bertani (LB) broth, respectively.

2.3. Measurement of antibacterial activity

Achacin was prepared from the mucus of the giant African snail, *A. fulica* Férussac, as described in [1]. Antibacterial activities of achacin and H_2O_2 were examined with a metabolic inhibition assay [1]. *S. aureus* or *E. coli* (approximately 10^6 colony forming units/ml) were cultured in 96-well plates in 100 μl of heart infusion bouillon medium, containing 1% glucose and test sample (dilution series of achacin or H_2O_2) for 16 h at 37°C. Antibacterial activity was determined by the lack of bacterial pellet after the incubation. The number of surviving cells was determined by the colony counting method as described previously [11].

2.4. Measurement of LAO activity

Xylenol orange assay was performed as described previously [12]. For the standard assay, the following reagents were added sequentially: 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange, and distilled water to a total volume of 180 μl. An aliquot (20 μl) of each sample was added to the mixture and incubated for 30 min at room temperature. LAO activity was determined by measuring the absorbance of Fe^{3+} (from oxidation of Fe^{2+} by H_2O_2)–xylenol orange complex at 550 nm. Blanks were prepared by replacing samples with 0.15 M NaCl.

2.5. Microscopic analysis of achacin- or H_2O_2-treated *E. coli*

E. coli (10^7 cells) were cultured in 1 ml of LB broth containing various concentration of achacin or H_2O_2. After incubation for 2 h at 37°C, bacterial suspensions were diluted 100-fold with sterilized water, mounted on glass slides, and stained with basic fuchsine. Morphology of *E. coli* was observed by light microscopy (Model BH-2-RFL, Olympus Optical Corp., Tokyo, Japan). Bacterial length was estimated by measuring the photo-images.

2.6. Analysis of bacteria-binding activity

Bacteria (10^8 cells) were cultured in 10 ml of LB broth for 2 h at 37°C. Bacteria were collected by centrifugation at 1000×*g* for 10 min,

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Abbreviations: LAO, L-amino acid oxidase; LB, Luria–Bertani; MIC, minimum inhibitory concentration; Neu5Ac, *N*-acetylneuraminic acid

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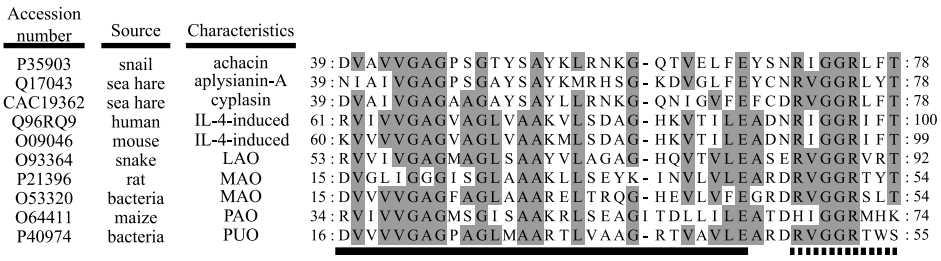

Fig. 1. Alignment of the amino acid sequences of the N-terminus of achacin and the amine oxidase consensus region. Solid underline indicates flavin-binding sites. Broken line indicates amino acids that are also conserved in flavin enzymes [29]. MAO, monoamine oxidase; PAO, polyamine oxidase; PUO, putrescine oxidase.

washed with 1 ml of phosphate-buffered saline (PBS) once, and suspended in 1 ml of 0.15 M NaCl. Then 50 μl of each bacterial suspension was added to 450 μl of either LB broth or 0.15 M NaCl both containing 5 μg/ml of achacin and/or *N*-acetylneuraminic acid (Neu5-Ac) and incubated for 2 h at 37°C. Bacterial proteins were collected by centrifugation of cells at 1000×*g* for 10 min, washing with 1 ml of PBS twice, and sonication at weak power in 6 M urea containing 1% Triton X-100. Protein concentrations were determined according to the method of Bradford [13]. Achacin in the lysate that had bound to bacteria was detected by SDS–PAGE followed by Western blot analysis.

2.7. SDS–PAGE and Western blot analysis

SDS–PAGE was performed according to the method of Laemmli [14] using 12.5% gels. Western blot analysis was done with anti-achacin polyclonal antibody as reported previously [11].

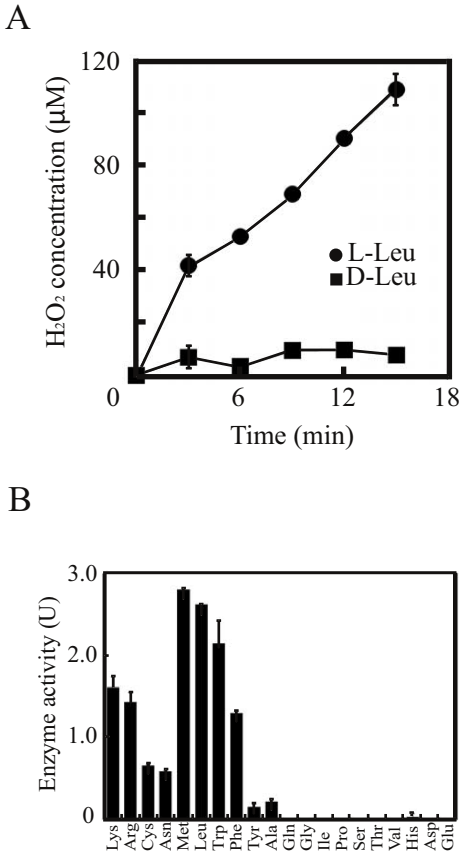

Fig. 2. LAO activity of achacin. A: Time- and amino acid type-dependent production of H_2O_2. L-Leu or D-Leu (10 mM) was used as substrate. B: L-Amino acid species-specific LAO activity of achacin. One unit (U) was defined as the amount that deaminizes 1 μmol of L-amino acid per min. Data represent mean ± S.D. (bar) of three independent experiments.

3. Results

3.1. LAO activity of achacin

Alignment of amino acid sequences showed that the characteristic flavin-binding site of amine oxidases was well conserved in achacin (Fig. 1). The oxidation activity of achacin was characterized for substrate specificity through analysis of various amino acids as substrates. Achacin showed high oxidase activity (2.62 U/mg) against L-Leu (Fig. 2A) similar to other LAOs [6,7,10]. Furthermore, achacin oxidized various L-type amino acids, whereas D-type amino acids were not oxidized, indicating that achacin is an LAO with broad substrate specificity for L-type amino acids (Fig. 2B).

3.2. Contribution of generated H_2O_2 to the antibacterial activity of achacin

Several LAOs from snake venoms show antibacterial and cytotoxic activities, and the H_2O_2 generated through the enzymatic reaction appears to be involved in these activities [6,15,16]. To examine the contribution of the generated H_2O_2 to the antibacterial activity of achacin, we measured the antibacterial activity of achacin against both Gram-positive (*S. aureus*) and -negative (*E. coli*) bacteria in the presence of several H_2O_2 scavengers. The antibacterial activity of achacin (2 μg/ml) was clearly inhibited by all scavengers tested (Table 1), suggesting that H_2O_2 is a mediator of the antibacterial activity.

We previously reported that achacin induces extensive filamentation of *E. coli* [4]. A similar morphological change was also observed in *E. coli* treated with a low concentration of H_2O_2 [17]. We compared the dose-dependent morphological changes in *E. coli* in the presence of achacin or H_2O_2 (Fig. 3). Cell filamentation was observed in H_2O_2-treated *E. coli* up to the minimum inhibitory concentration (MIC) (Fig. 3A,B); however, no obvious changes were observed at higher concen-

Table 1
Inhibitory effect of H_2O_2 decomposers on antimicrobial activity of achacin

Substance	Inhibitory concentration[a]	
	S. aureus	*E. coli*
MnO_2	0.5 mg/ml[b]	0.5 mg/ml[b]
$FeSO_4$	250 μM	250 μM
Catalase	0.03 U[d]	0.25 U
HRP[c]	0.02 U	0.08 U

[a]Incubated with 2 μg/ml of achacin under MIC measurement condition.
[b]Concentration as a suspension.
[c]HRP: horseradish peroxidase.
[d]U: unit.

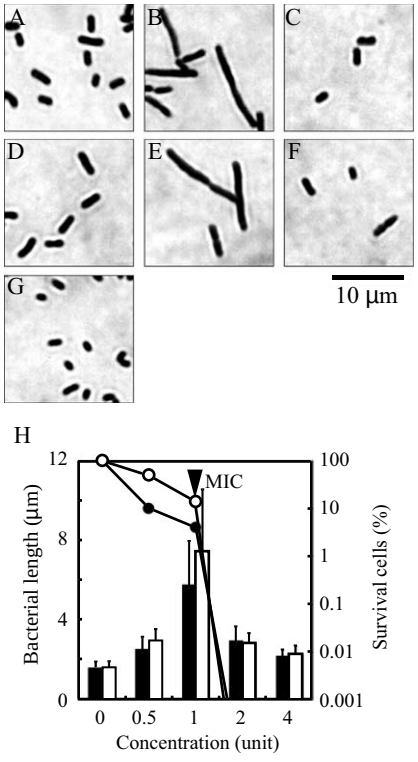

Fig. 3. Comparison of the morphological changes in achacin- and H_2O_2-treated *E. coli*. Morphology of achacin-treated (A–C) or H_2O_2-treated (D–F) *E. coli* was observed by light microscopy. MICs of achacin and H_2O_2 were each defined as 1 unit. A–C: *E. coli* treated with 0.5, 1, and 4 units of achacin, respectively. D–F: *E. coli* treated with 0.5, 1, and 4 units of H_2O_2, respectively. G: Physiological saline control. H: Statistical representation of morphological changes and survival of *E. coli*. The bacterial lengths of *E. coli* treated with achacin (solid bars, $n=40$) and H_2O_2 (open bars, $n=40$) were measured. The numbers of *E. coli* surviving after treatment with achacin (solid circles) and H_2O_2 (open circles) were determined by the colony counting method [3]. Arrowhead indicates MIC.

trations (four-fold higher than the MIC) (Fig. 3C). Similar morphological changes were also observed in achacin-treated *E. coli* (Fig. 3D–F). Statistical analyses revealed that the peaks of the achacin and H_2O_2 filamentation activities corresponded to the MICs and appeared to accompany the acute reduction in the number of surviving cells (Fig. 3H). These data indicate that the morphological changes observed in achacin-treated *E. coli* are due primarily to the generated H_2O_2.

To confirm our observation, we examined the time-dependent H_2O_2 productivity of achacin under the conditions we used for measurement of antibacterial activity. At MICs for *S. aureus* (0.2 μg/ml) and *E. coli* (2 μg/ml), achacin produced 0.2 and 0.4 mM H_2O_2, respectively (Table 2). However, the MICs of H_2O_2 that we determined under the same conditions were 0.7 mM (against *S. aureus*) and 1 mM (against *E. coli*) (Table 2). This inconsistency suggests that achacin has a novel function that allows for maximal antibacterial action.

3.3. Bacteria-binding activity of achacin

The growth-phase-specific antibacterial activity and bacterial plasma membrane localization of achacin were reported previously [3,4]. Thus, we hypothesized that achacin binds to bacterial plasma membrane in a growth-phase-specific manner

Table 2
Comparison of MICs between achacin and H_2O_2

Bacteria	MICs	
	Achacin	H_2O_2
S. aureus	0.2 μg/ml (0.2 mM)[a]	0.7 mM
E. coli	2 μg/ml (0.5 mM)[a]	1 mM

[a]Incubation with achacin was up to 24 h. Maximal H_2O_2 concentration at indicated achacin concentrations was detected at approximately 2 h incubation.

and that this allows achacin to kill bacteria with a lower concentration of H_2O_2. To confirm this hypothesis, we examined the bacteria-binding activity of achacin by Western blotting (Fig. 4). Achacin incubated with culture medium (growth condition) showed significant bacteria-binding activity against *S. aureus* and *E. coli* (Fig. 4A, lanes 2 and 5). In contrast, achacin incubated with physiological saline (rest condition) showed only slight binding (Fig. 4A, lanes 1 and 4), indicating that achacin preferentially recognizes the growth-phase bacteria. In addition, the binding activity was higher against *S. aureus* than against *E. coli*, which is consistent with the observed differences in MICs between *S. aureus* (0.2 μg/ml)

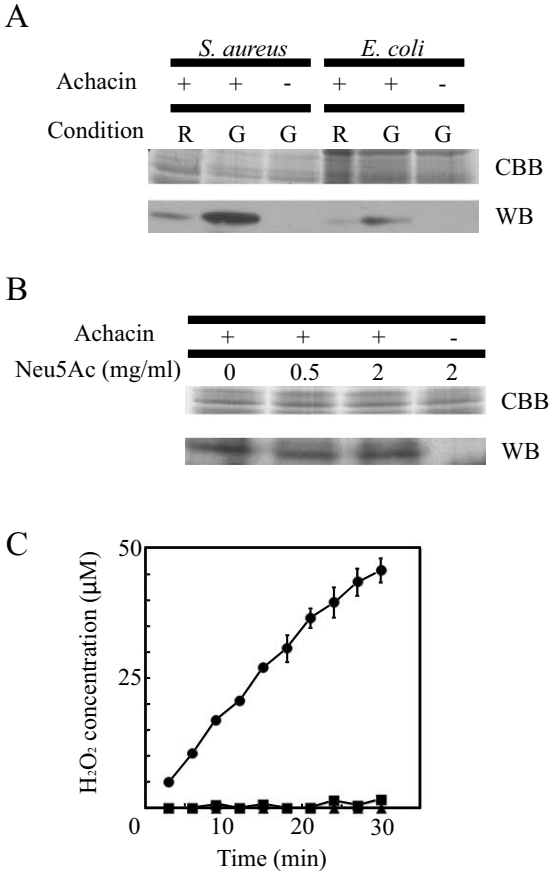

Fig. 4. Bacteria-binding activity of achacin. A: Bacteria-binding activity of achacin against both Gram-positive (*S. aureus*) and -negative (*E. coli*) bacteria under rest conditions (R) and growth conditions (G). B: Effect of sialic acid on the bacteria-binding activity of achacin against *S. aureus* was examined in the presence of Neu5Ac. C: Inhibitory effect of Neu5Ac on the LAO activity of achacin. The LAO activity of achacin was measured in the presence (square, 0.5 mg/ml; triangle, 2 mg/ml) and absence (circle) of Neu5Ac. CBB, Coomassie brilliant blue staining; WB, Western blotting with anti-achacin antibody.

and *E. coli* (2 μg/ml). These results indicate that binding to bacteria is important for the antibacterial activity of achacin.

Aplysianin E, the putative processed form of aplysianin A that shows homology to achacin [18], also has antibacterial and antitumor activities [19,20]. The tumor lysis activity of aplysianin E was inhibited by Neu5Ac, suggesting that recognition of a sugar moiety is a key step in this process [19]. In a preliminary study, we also found that the antibacterial and cytotoxic activities of achacin were inhibited by Neu5Ac (data not shown). Therefore, we investigated whether Neu5Ac affects the bacteria recognition or LAO activity of achacin (Fig. 4B,C). Up to 2 mg/ml of Neu5Ac did not block the bacteria-binding activity of achacin (Fig. 4B, lane 3), and higher concentrations of Neu5Ac inhibited bacterial growth (data not shown). In contrast, Neu5Ac inhibited the LAO enzyme activity (Fig. 4C); this inhibition should be sufficient to diminish the antibacterial activity of achacin.

4. Discussion

In the present study, we show that the antibacterial activity of achacin is due to H_2O_2 generated by LAO activity. However, the concentration of achacin-generated H_2O_2 in the culture medium was not sufficient to inhibit bacterial growth. Growth-phase-specific bacteria binding appeared to play an important role in the antibacterial activity of achacin. In fact, Suhr et al. [21] reported that LAO from snake venom bound to the cell surface in a cell-selective manner. This selectivity is thought to lead to the cell-specific cytotoxicity of the LAO through locally increased H_2O_2 [21]. These data suggest that when snails are infected by pathogens, achacin should bind to the plasma membranes of those that are proliferating. Achacin may attack pathogens during other growth phases by increasing the local concentration of H_2O_2 so as not to harm neighboring host cells.

Despite many studies, the substance or structure recognized by achacin has not yet been determined. Neu5Ac, which is a sialic acid, is reported to act as a potent inhibitor of the antitumor activity displayed by some antitumor glycoproteins isolated from mollusks [19,22,23]. However, our data showed that Neu5Ac inhibited the antibacterial activity of achacin through inhibition of the LAO activity and not through inhibition of the bacteria-binding activity. Thus, recognition of sugar moieties should not be essential for the antibacterial activity of achacin. Cytotoxic cyplasin was recently isolated from the mucus of the European sea hare, *A. punctata* [24], and it shows significant amino acid sequence similarity to achacin. The cytotoxic action of cyplasin is preferential to growing cells [24], as is the antibacterial activity of achacin. In mollusks, these antibacterial and cytotoxic glycoproteins are localized to mucus [1,24], albumen gland, egg, purple fluid, and coelomic fluid [23]. Because these tissues and fluids act as the organism's first line of defense against the external environment, the putative biological functions of these glycoproteins might be as defense factors.

In higher vertebrates, a fish LAO (AIP, [7,15]), interleukin 4 (IL-4)-induced gene (*Fig1*, [25–27]), and mouse milk LAO [28] are reported to work as host defense factors. Thus, LAOs, which are widely distributed in living organisms, appeared to be important in both vertebrate and invertebrate host defenses.

In the present study we showed that achacin preferentially recognizes and binds to growth-phase bacteria to kill them with H_2O_2 generated locally. Furthermore, this selective toxicity suggests that achacin might be useful as a multifunctional chemotherapeutic agent because it may be more toxic to proliferating pathogens than to host cells or normal flora, which have slower growth rates.

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