Specificity and Enzyme Kinetics of the Quorum-quenching
N-Acyl Homoserine Lactone Lactonase (AHL-lactonase)*

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N-Acyl homoserine lactone (AHL) quorum-sensing signals are the vital elements of bacterial quorum-sensing systems, which regulate diverse biological functions, including virulence. The AHL-lactonase, a quorum-quenching enzyme encoded by aiiA from Bacillus sp., inactivates AHLs by hydrolyzing the lactone bond to produce corresponding N-acetyl homoserines. To characterize the enzyme, the recombinant AHL-lactonase and its four variants were purified. Kinetic and substrate specificity analysis showed that AHL-lactonase had no or little residue activity to non-acetyl lactones and non-cyclic esters, but displayed strong enzyme activity toward all tested AHLs, varying in length and nature of the substitution at the C3 position of the acyl chain. The data also indicate that the amide group and the ketone at the C3 position of the acyl chain of AHLs could be important structural features in enzyme-substrate interaction. Surprisingly, although carrying a 104HXH109 short sequence identical to the zinc-binding motif of several groups of metallohydrolytic enzymes, AHL-lactonase does not contain or require zinc or other metal ions for enzyme activity. Except for the amino acid residue His-104, which was shown previously to not be required for catalysis, kinetic study and conformational analysis using circular dichroism spectrometry showed that substitution of the other key residues in the motif (His-106, Asp-108, and His-109), as well as His-169 with serine, respectively, caused conformational changes and significant loss of enzyme activity. We conclude that AHL-lactonase is a highly specific enzyme and that the 104HXH109–H109 of AHL-lactonase represents a novel catalytic motif, which does not rely on zinc or other metal ions for activity.

Many host-associated bacteria produce, release, and respond to small signal molecules to monitor their own population density and control the expression of specific genes in response to change in population density. This type of gene regulation, which controls diverse biological functions including virulence and biofilm formation, is known as quorum-sensing (QS)1 (1–4). In general, each individual bacterial cell produces a basal level of QS signals. The signals accumulate to a threshold concentration as the cells proliferate and interact with their cognate transcription factors to activate gene expression. Several groups of QS signals have been identified. Among them, N-acetyl homoserine lactones (AHLs) comprise a family of QS signals identified in many Gram-negative bacteria, in particular, Proteobacteria. Different bacterial species may produce different AHLs, which vary in length and substitution of the acyl chain but maintain the same homoserine lactone moiety (1, 3, 4). These structural variations could constitute the basis of signaling specificity of AHL molecules (5, 6).

The AHL-dependent QS system has drawn considerable attention over the last 10 years, as it is involved in the regulation of diverse and important biological functions, in particular, the virulence gene expression in a range of animal (including human) and plant bacterial pathogens such as Erwinia carotovora and Pseudomonas aeruginosa (7–14). Being a key attribute that determines virulence gene expression in pathogenic bacteria, the AHL signaling system has been regarded as a promising target for developing novel approaches to controlling bacterial infections. Several anti-QS mechanisms have been identified in recent years. AHL antagonists were found to interfere with bacterial QS signaling by inducing accelerated degradation of the AHL-dependent transcription factor (15–16). Two groups of enzymes, i.e. the acyl-homoserine lactonase (AHL-lactonase) and acyl-homoserine lactone acylase (AHL-acylase), which degrade AHL by hydrolyzing, respectively, the lactone bond and the amide linkage (Fig. 1), were identified from numerous bacterial isolates (17–24). Genetically modified E. carotovora and P. aeruginosa expressing AHL-lactonase or AHL-acylase showed decreased production of virulence factors and attenuated virulence (17, 18, 21, 22). Plants expressing AHL-lactonase quenched pathogen QS signaling and showed significantly enhanced resistance to E. carotovora infection (18). These findings highlight the promising potential for establishing a generic “quorum-quenching” approach to control bacterial infections, that is, to paralyze the quorum-sensing of bacterial pathogens through inactivation of QS systems (18, 25).

AHL-lactonase appears to be a potent enzyme. It works well at physiologically relevant concentrations of AHL signals and hydrolyzes the four tested AHLs (C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, and 3-oxo-C12-HSL) effectively (18). The enzyme contains a conserved short sequence 104HXH109, which is identical to the Zn2+–binding motif of several metallohydrolases (26–28). Within the sequence, three amino acid residues, His-106, Asp-108, and His-109, plus His-169, which is also conserved in the metallohydrolases (17), have proven to be essential for the AHL-lactonase activity (17, 19). It is not clear whether Zn2+ or other ions are required for the catalytic function of AHL-lactonase. Little is known about its substrate specificity and enzyme properties. In this study, we have investigated the catalytic activity of AHL-lactonase against a range of...
AHL derivatives and related compounds. To probe the enzymatic mechanism, the metal ion composition of the enzyme and effect of ions on enzyme activity have also been determined. Furthermore, four AHL-lactonase variants deficient in enzyme activity have been purified for kinetic assay and conformational analysis using circular dichroism spectrometry in an attempt to reveal the structural features governing substrate–enzyme interaction and catalytic efficiencies.

EXPERIMENTAL PROCEDURES

Synthesis of AHLs and Derivatives—AHLs were synthesized as described (6), except that 3-hydroxybutanoyl-L-homoserine lactone was purchased from Quorum Sciences Inc. The acyl homoserines (Acyl-HSs) were prepared by incubating the corresponding acyl homoserine lactones in 1:1 ratio of 1 M NaOH/dimethyl sulfoxide (v/v) for 12 h at room temperature. The solution was neutralized to pH 6.5 with 1 M NaH₂PO₄ and then dried under vacuum. These synthetic AHLs and Acyl-HSs were purified using silica gel column chromatography and C₁₈ reversed-phase HPLC and confirmed structurally by ¹H NMR spectroscopy and electrospray ionization mass spectrometry. Other reagents were purchased from Sigma-Aldrich unless otherwise stated.

Purification of AHL-lactonase and Its Variants—The aiaA gene encoding AHL-lactonase and its variants H106S, D108S, H109S, and H169S contained in the pGEM-7Zf(+) vector were amplified, respectively, by PCR using forward primer 5′-ATCGTACCCATGCAGTAAAGAAGCTTTATTTGC-3′ and reverse primer 5′-GTCGAATTCTCTACACTGATCTTCATGCACG-3′ (17). The PCR products were digested by BamHI and EcoRI and fused in-frame to the glutathione S-transferase (GST) gene under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible tac promoter in GST fusion vector pGEX-2T (Amersham Biosciences). The constructs were verified by DNA sequencing.

Escherichia coli containing different constructs was cultured at 30 °C in 4-liter LB medium containing 100 μg/ml ampicillin. The GST-AHL-lactonase fusion protein and its variants were expressed by the addition of IPTG to a final concentration of 0.5 mM after the optical density of bacterial culture reached 0.4–0.5 at 600 nm; the culture was then incubated at 28 °C overnight. The cells, harvested by centrifugation and resuspended in 1× PBS buffer (pH 7.4), were disrupted twice with a chilled French pressure cell at 2000 p.s.i. Cell debris was removed by centrifugation (11,000 × g for 30 min, 4 °C). The supernatant was added to a glutathione-Sepharose 4B affinity column (Amersham Biosciences). The GST fusion proteins were bound to the affinity matrix, and AHL-lactonase and its variants were separated from GST by digestion with the protease thrombin overnight at room temperature. After digestion, the eluates containing AHL-lactonase were combined, and the purity was analyzed by 10% SDS-PAGE. The purified AHL-lactonase and its variants were stored at −80 °C prior to use. The enzyme concentration was determined by UV spectrophotometry at 280 nm based on their corresponding molar extinction coefficients (for example, ε₅₇₈ nm = 19707 M⁻¹ cm⁻¹).

Metal Ion Determination—The metal ion composition of AHL-lactonase was determined on a PerkinElmer Life Sciences SCIEX ELAN 6100 inductively coupled plasma mass spectrometry (ICP-MS). Mass discrimination, auxiliary argon, and coolant gas flow rates were controlled automatically by the instrument. The other operating conditions were adjusted to maximize the signal for analyte ion using standard solutions.

Circular Dichroism Spectroscopy—Far-UV CD study of AHL-lactonase and its variants was carried out on a JASCO J-810 spectropolarimeter at 22 °C in the wavelength range between 185 and 260 nm under constant nitrogen flow, using 1-mm path length quartz cells. The spectra were derived from an average of five scans recorded at 50 mm⁻¹, along with a 1-s time constant. Each spectrum was corrected against blank, smoothed, and analyzed using the software package provided by JASCO. The instrument was regularly calibrated using ammonium d(+)−10-camphorsulfonate following the manufacturer’s recommendations. Baseline was corrected with 1× PBS buffer in the absence of enzyme. The fraction of secondary structure was estimated using the method described previously (28).

Enzyme Kinetics and Specificity of AHL-lactonase—To determine enzyme kinetics, AHL-lactonase was added at a final concentration of 1 μM to AHL solution (0.3–20 μM) in 0.1 M phosphate buffer (pH 7.4) with a final volume of 96 μl. The reactions were incubated at 22 °C, stopped by adding 24 μl of 10% SDS, and subjected to HPLC analysis. The residual AHL and its hydrolysis product were quantified by HPLC. All experiments were performed in triplicate, and all velocities were determined at time points at which no more than 10% of the substrate had been consumed. The kcat and Km values were calculated based on Michaelis-Menten equation. The enzyme specificity was determined by the same procedure, except that the substrate and enzyme concentrations were fixed at 3 μM and 0.5 μM, respectively, and the reaction time was 10 min.

Substrate Binding Assay—To determine the substrate binding ability of the enzymes, 2-μl solutions containing 20 μM enzyme and 10–200 μM 3-oxo-C8-HSL were transferred to Centricon-10 tubes (Amicon, Milipore) and centrifuged at 5000 × g until the volume of concentrate was ~60–80 μl. The concentrations of 3-oxo-C8-HSL in the final concentrate and the filtrate were quantified separately by bioassay analysis.

RESULTS

Purification and Properties of AHL-lactonase—The GST-AiiA fusion protein was expressed in E. coli following IPTG induction and purified by routine GST affinity chromatography procedure. The recombinant AiiA (AHL-lactonase), which has two extra amino acid residues (Gly and Ser) at the N terminus than does the native AiiA, was separated from GST by thrombin digestion. The recombinant enzyme was purified 86-fold with a yield of ~73% of the total proteins. The SDS-PAGE
analysis indicates that the purity of the obtained recombinant AHL-lactonase (7 μg loaded) should be >98.5%, because staining with Coomassie Brilliant Blue R-250, which can detect as little as 0.1 μg of protein, did not reveal other protein bands (Fig. 2). The SDS-PAGE analysis showed the size of the purified AHL-lactonase enzyme is ~28 kDa, which is consistent with the predicted molecular mass of 28,036 Da (17).

The optimal pH for AHL-lactonase activity was examined using 3-oxo-C8-HSL as substrate. AHL-lactonase activity, enhanced with pH increasing from 6 to 8, reached the maximum at pH 8, then declined slightly at pH 9 (Fig. 3A). The potential interference of non-enzymatic pH-dependent lactone hydrolysis was precluded by analysis of the controls in which corresponding AHL was incubated in the same reaction buffer without the enzyme. The enzyme appeared unstable at low pH, which was confirmed by CD analysis as described in the next section; no or little activity was detected when pH was adjusted to 5 or below.

AHL-lactonase exhibited excellent thermal stability at temperatures below 37 °C, and the purified enzyme, kept at 4 and 21 °C for 10 days, still maintained >99% activity. But the enzyme is less stable at higher temperatures; its activity decreased sharply after incubation for 2 h at >45 °C (data not shown). The effect of temperature on enzyme catalytic activity was analyzed using 3-oxo-C8-HSL as substrate. Up to a maximum of 37 °C, the enzyme activity displayed typical temperature dependence as shown in the Arrhenius plot in Fig. 3B, whereas at 45 °C enzyme inactivation was noticed. In the range between 6–37 °C, the activation energy $E_a$ was calculated to be 52.4 kJ·mol$^{-1}$ from the slope ($E_a/R$) of the graph. The enthalpy $\Delta H^*$ and entropy $\Delta S^*$ of activation were calculated to be 49.9 kJ·mol$^{-1}$ and $-53.7$ J·mol$^{-1}$·K$^{-1}$, respectively. The free energy $\Delta G^*$ of activation at 25 °C was calculated to be 65.9 kJ·mol$^{-1}$. These thermodynamic parameters were calculated by the equations $\Delta G^* = -RT \ln(k_b h/k_B T)$, $\Delta H^* = E_a - RT$, and $\Delta S^* = (\Delta H^* - \Delta G^*)/T$, where $k_b$, $h$, and $R$ are Boltzmann, Planck, and universal gas constants, respectively.

Several metal ions, including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$, showed no effect on enzyme activity at 0.2 and 2 mM, respectively (Fig. 3C). On the other hand, AHL-lactonase was partially inhibited by Cr$^{3+}$ (72%), Pb$^{2+}$ (67%), and Fe$^{3+}$ (48%) at 2 mM and completely inhibited by Cu$^{2+}$ and Ag$^{+}$ at 0.2 mM, possibly due to reaction of sulfhydryl groups of the enzyme with Cu$^{2+}$ and Ag$^{+}$ (30). The chelating reagents such as EDTA, 2,2'-bipyridine, and o-phenanthroline at a concentration of 2 mM had no effect on enzyme activity.

**The Conformational Structure of AHL-lactonase Is pH-dependent**—The AHL-lactonase encoded by the aiiA gene is an acidic protein with its isoelectric point at 4.7 (17). The pH-dependent pattern of the enzyme activity shown in Fig. 3A suggests that the electrostatic interactions between the charged amino acid residues of AHL-lactonase could play important roles in maintenance of the overall structural conformation and the local electrostatic potentials at the catalytic center, which is critical for enzyme activity. We used CD spectrometry to determine the effect of pH on AHL-lactonase conformational structure. Fig. 4 shows that pH has a drastic effect on the conformational structure of AHL-lactonase. The asymmetric conformational structure of AHL-lactonase remained unchanged in pH ranging from 7 to 9 and slightly changed at pH 6, but significantly changed at pH 5.5 and completely lost at pH 5. The data are consistent with the pH-dependent enzyme activity pattern of AHL-lactonase (Fig. 3A).
AHL-lactonase Is Not a Metalloenzyme—Sequence alignment suggested that AHL-lactonase contains a motif similar to the Zn$^{2+}$ binding motif of metalloenzymes (17). To determine whether it is a metalloenzyme, we measured Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$ metal ion contents in AHL-lactonase by ICP-MS. Surprisingly, no metal ion was detectable except for a trace mount of Zn$^{2+}$. The Zn content was $-0.08$ mol per mol of protein, inconsistent with the notion that it is a metallohydrolase. Moreover, the zinc-free AHL-lactonase, generated by treatment with cleaving reagent EDTA and dialysis and confirmed by ICP-MS analysis, maintained the same level of enzymatic activity as the untreated enzyme.

AHL Hydrolysis by AHL-lactonase—To compare the enzyme activity of AHL-lactonase toward different AHLs, we synthesized and tested 10 AHL molecules. These AHLs differ in acyl chain length and substitution at the C9 position of the acyl chain (Fig. 5). AHL-lactonase showed an excellent ability to accommodate these structural differences regardless of which AHL was used as a substrate. More than 80% of AHL was degraded by AHL-lactonase in 1:2500 molar ratio of enzyme to substrate in the first 10 min of reaction, and only one new fraction was found after the enzyme reaction by HPLC analysis. The new fraction showed the identical HPLC retention time of the substrate in the first 10 min of reaction, and only one new product failed to show any activity even the 1500-fold) in biological activity of AHL-lactonase led to a sharp decrease (within these narrow ranges, the enzyme showed higher affinity ($K_m$), slower hydrolysis rate ($k_{cat}$), and stronger catalytic efficiency ($k_{cat}/K_m$) toward the AHLs with longer acyl side chain than the shorter derivatives. Additionally, the enzyme displayed higher $k_{cat}$ and $k_{cat}/K_m$ values against the AHLs with fully reduced acyl chains than their corresponding derivatives containing 3-oxo substitution (Table II).

Kinetic Analysis of AHL-lactonase—Hydrolysis kinetics was determined by plotting velocity versus substrate concentration. The $k_{cat}$ and $K_m$ values were calculated by fitting the data to the Michaelis-Menten equation (Table II). AHL-lactonase showed comparable catalytic activity against a range of structurally different AHLs with $k_{cat}$ and $K_m$ values ranging 20.22–37.63 s$^{-1}$ and 1.43–7.51 mM, respectively, at pH 7.4 and 22 °C. Within these narrow ranges, the enzyme showed higher affinity ($K_m$), slower hydrolysis rate ($k_{cat}$), and stronger catalytic efficiency ($k_{cat}/K_m$) toward the AHLs with longer acyl side chain than the shorter derivatives. Additionally, the enzyme displayed higher $k_{cat}$ and $k_{cat}/K_m$ values against the AHLs with fully reduced acyl chains than their corresponding derivatives containing 3-oxo substitution (Table II).

Kinetics and Circular Dichroism Analysis of AHL-lactonase Variants Deficient in Enzyme Activity—The catalytic mechanism of AHL-lactonase has not yet been characterized. The previous study showed that AHL-lactonases share a conserved motif, $^{106}$HXDH$^{109}$–H$^{169}$, which is similar to the zinc-binding motif of several metallohydrolases (17, 27, 28). However, the metal ion content analysis indicates that AHL-lactonase is unlikely to be a metallohydrolase. To study the roles of these conserved amino acid residues in enzyme catalysis, we prepared four GST fusion protein constructs to express and purify four AHL-lactonase variants (H106S, D108S, H109S, and H1169S). SDS-PAGE analysis showed that the AHL-lactonase variants were similar to AHL-lactonase in protein expression, except that the replacement of Asp-108 with serine (D108S) resulted in a much higher level of expression (Fig. 2). The four AHL-lactonase variants were purified and separated from GST by digestion with site-specific protease thrombin.

To probe the function of the conserved $^{106}$HXDH–H$^{169}$ motif, we analyzed the enzyme kinetics of four AHL-lactonase variants. Table III shows that single replacement of Asp-108 and His-109 by serine, respectively, led to complete loss of enzyme activity, whereas the mutations of His-106 and His-169 to serine resulted in loss of activity by $-47\%$. The data are consistent with the previous results based on total soluble protein assay (17, 19). The $K_m$ value for the variants H106S and H169S were 10.8 and 13.5 mM, respectively, using 3-oxo-C8-HSL as substrate. Compared with the $K_m$ value of AHL-lactonase (Table II), the results show that the enzymatic affinity was substantially decreased after replacement of His-106 and His-169 with serine. Kinetic analysis also found that the catalytic efficiency of H106S and H169S, represented by the $k_{cat}/K_m$ values, was 9 and 12 times lower than that of the wild type enzyme (Table III), respectively.

CD spectroscopy was used to monitor the potential protein structural changes caused by serine substitution of the key amino acid residues. The CD spectrum of AHL-lactonase showed an intense peak of negative ellipticity at 202 nm with a small shoulder peak at 222 nm (Fig. 6), indicating a large unordered contribution and a small but detectable contribution of $a$-helical structure. In contrast, the CD spectra of the four variants differed from those of their wild type enzyme. Their maximum CD absorbance shifted from 202 nm to $-206$ nm,
Fig. 5. Substrate specificity of AHL-lactonase. Incubations were carried out for 10 min at 22 °C in 60-μl reaction mixtures containing 3 mM indicated substrate, 0.5 μM AHL-lactonase, and 0.1 M phosphate buffer, pH 7.4. The residual substrate was quantified by HPLC. Data are the means of 3–6 measurements. The activity toward 3-oxo-C8-HSL (9.06 μmol/min·mg) was defined as 100%.

| Substrates     | Structure | Activity (%) |
|----------------|-----------|--------------|
| 1. AHLs        |           |              |
| 3-oxo-C4-HSL   |           | 87.5         |
| 3-oxo-C6-HSL   |           | 91.8         |
| 3-oxo-C8-HSL   |           | 100          |
| 3-oxo-C10-HSL  |           | 109.1        |
| 3-oxo-C12-HSL  |           | 97.2         |
| C4-HSL         |           | 110.9        |
| C6-HSL         |           | 124.5        |
| C8-HSL         |           | 116.9        |
| C10-HSL        |           | 112.4        |
| 3-HO-C4-HSL    |           | 83.6         |
| 2. Other lactones |         |              |
| γ-Butyrolactone|           | 2.49         |
| δ-Valerolactone|           | 2.10         |
| ε-Caprolactone |           | 3.81         |
| L-Homoserine lactone | | 6.72 |
| γ-Valerolactone|           | 2.67         |
| γ-Decanolactone |           | 1.85         |
| 3. Esters      |           |              |
| Ethyl acetate  |           | 0            |
| Phenyl acetate |           | 0            |
| p-Nitrophenyl acetate | | 0 |
| α-Naphthyl acetate |       | 0            |
and the intensities of the negative peaks at 222 nm were increased, indicating that all variants have higher α-helix contents and lower random coil contents than the wild type AHL-lactonase (Fig. 6 and Table III). The data suggest that the single amino acid substitution of the key residues involved in enzyme activity caused a conformational change, resulting in reduction or loss of the enzyme activity. This conformational change caused by the single amino acid substitution was also reported in other proteins, such as the human apolipoprotein C-III (35). Interestingly, the CD spectra of H106S and H169S and those of D108S and H109S are similar, respectively, in particular at the structural characteristic negative bands around 206 and 222 nm (Fig. 6). This is in agreement with the enzyme activity data that showed that substitution of His-106 and His-169, respectively, caused a similar level of decrease in enzyme activity, whereas a change in either Asp-108 or His-109 has the same detrimental effect (Table III). The data suggest that Asp-108 and His-109 might share a similar functional role, whereas His-106 and His-169 could be another pair with equivalent enzymatic contributions.

Substrate binding ability was determined by centrifugal ultrafiltration (36). The method is based on centrifugal ultrafiltration through a membrane with a molecular mass cutoff intermediate between that of the ligand and that of the target protein. The amount of bound ligand is calculated by subtracting the (free) ligand in the ultrafiltrate fraction from the total ligand added to enzyme solution. D108S and H109S showed no binding ability to substrate 3-oxo-C8-HSL, and the added AHL was quantitatively recovered in ultrafiltrate. The binding ability of H106S and H169S could not be determined by this method, because their digestion speeds surpassed the time required for completion of ultrafiltration.

**DISCUSSION**

AHL-lactonase has been used previously to demonstrate that quenching bacterial quorum sensing is a promising strategy for preventing and controlling bacterial infections (17, 18). The data presented here show that the AHL-lactonase encoded by the aiaA gene from Bacillus sp. 240 is a potent enzyme, demonstrating strong catalytic activity and remarkable substrate specificity against different AHL signal molecules (Table II and Fig. 5). The results also showed that AHL-lactonase is not a metallohydrolase enzyme, despite containing an obvious short stretch of sequence that resembles the zinc-binding motif HX–HXX–H of several groups of the metallohydrolase family (17, 26–28).

To determine the substrate spectrum of AHL-lactonase and the structural features of AHBs that are important for enzyme-substrate interaction, we synthesized nine AHB compounds and obtained 3-hydroxybutanoyl L-homoserine lactone from a commercial source. By quantification of the residual AHB and its hydrolysis product, we determined the relative enzyme activity of AHL-lactonase on different AHB derivatives. The data indicate that AHL-lactonase has a broad substrate spectrum and digests efficiently all of the 10 AHB compounds used in this study, regardless of the length and substitution of the acyl chain (Fig. 5). However, within a narrow window of variations, the enzyme activity is somewhat affected by the length and substitution of the acyl chain of the substrates. The best substrates of AHL-lactonase are C6-HSL among the tested AHL signals (Fig. 6). The data were means from triplicate experiments.

### TABLE I

| AHL hydrolysis products | Minimum concentration for activity | Decreased activity (times) compared with the corresponding AHL |
|------------------------|-----------------------------------|----------------------------------------------------------|
| 3-Oxo-C4-HS            | ND                                | ND                                                       |
| 3-Oxo-C6-HS            | 500                               | 1667                                                     |
| 3-Oxo-C8-HS            | 50                                | 2500                                                     |
| 3-Oxo-C10-HS           | 300                               | 1500                                                     |
| 3-Oxo-C12-HS           | 1000                              | 1667                                                     |
| C4-HS                  | ND                                | ND                                                       |
| C6-HS                  | ND                                | ND                                                       |
| C8-HS                  | 1000                              | 2000                                                     |
| C10-HS                 | 2000                              | 2000                                                     |
| 3-HO-C4-HS             | ND                                | ND                                                       |

### TABLE II

**Kinetic parameters of AHL-lactonase against AHBs**

| AHBs       | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|-----------|-------|---------------|
| 3-Oxo-C4-HSL | 28.63     | 4.07  | 7.03          |
| 3-Oxo-C6-HSL | 22.68     | 2.95  | 7.69          |
| 3-Oxo-C8-HSL | 22.17     | 2.28  | 9.72          |
| 3-Oxo-C10-HSL | 20.22     | 1.43  | 14.1          |
| 3-Oxo-C12-HSL | ND        | ND    | ND            |
| C4-HSL     | 37.63     | 5.11  | 7.36          |
| C6-HSL     | 35.67     | 3.83  | 9.31          |
| C8-HSL     | 27.53     | 2.61  | 10.5          |
| C10-HSL    | ND        | ND    | ND            |
| 3-HO-C4-HSL | 29.30     | 7.51  | 3.90          |

### TABLE III

**Relative enzyme activity and the secondary structure contents of AHL-lactonase and variants**

| Enzymes     | Activity | α-Helix | β-Sheet | β-Turn | Random |
|-------------|----------|---------|---------|--------|--------|
| AHL-lactonase | 100      | 6.5     | 48.2    | 0      | 45.3   |
| H106S       | 53.50    | 16.5    | 48.7    | 2.7    | 32.1   |
| H169S       | 53.05    | 14.8    | 50.8    | 4.9    | 29.5   |
| D108S       | 0        | 13.3    | 52.5    | 0      | 34.2   |
| H109S       | 0        | 13.6    | 51.1    | 0      | 35.3   |

*ND indicates that no activity was detected in the concentration range from 10–10,000 μM.

*3-Hydroxybutanoyl L-homoserine.
of AHLs is not required for enzyme-substrate interaction.

AHL-lactonase appears to be highly specific to AHLs, showing only residual activity against l-homoserine lactone and non-homo-
serine lactones (Fig. 5). The enzyme did not hydrolyze non-
cyclic esters. Among the tested non-acyl lactones, it is interesting to note that the enzyme showed slightly higher residue activity
against l-homoserine lactone than γ-decanolactone, which has a
6-carbon alkane chain (Fig. 5). This observation, together with
the finding that AHL-lactonase has high catalytic activity
against all of the tested AHL derivatives regardless of the substi-
tuation status at C3 position, suggests that the amide group
and the ketone at the C1 position of the acyl chain of AHLs could
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