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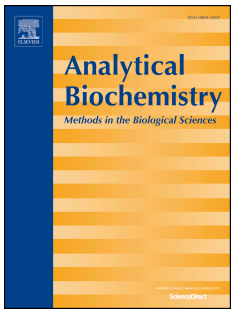


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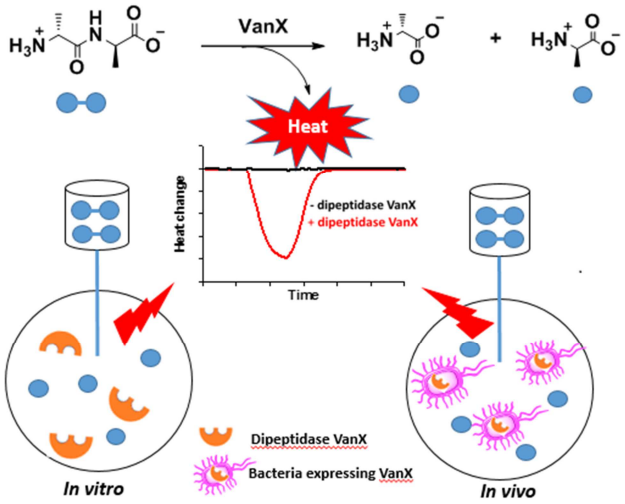


A novel ITC approach for continuous assay of the activity and inhibition of VanX enzyme *in vitro* and in living bacterial by using D-Ala-D-Ala as the substrate.

# Real-time monitoring of D-Ala-D-Ala dipeptidase activity of VanX in living bacteria by isothermal titration calorimetry

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**Abstract**

The D,D-dipeptidase enzyme VanX is the main cause of vancomycin resistance in gram-positive bacteria because of hydrolysis of the D-Ala-D-Ala dipeptide used in cell-wall biosynthesis. Continuous assay of VanX has proven challenging due to lack of a chromophoric substrate. Here, we report a direct approach for continuous assay of VanX *in vitro* and *in vivo* from hydrolysis of D-Ala-D-Ala, based on the heat-rate changes measured with isothermal titration calorimetry (ITC). With the ITC approach, determination of kinetic parameters of VanX hydrolyzing D-Ala-D-Ala and the inhibition constant of D-cysteine inhibitor yielded $K_M$ of 0.10 mM, $k_{cat}$ of 11.5 $s^{-1}$, and $K_i$ of 18.8 μM, which are consistent with the data from ninhydrin/Cd(II) assays. Cell-based ITC studies demonstrated that the VanX expressed in *E. coli* and in clinical strain VRE was inhibited by D-cysteine with $IC_{50}$ values of 29.8 and 28.6 μM, respectively. Also, the total heat from D-Ala-D-Ala (4 mM) hydrolysis decreases strongly (in absolute value) from 1.26 mJ for VRE to 0.031 mJ for *E. faecalis*, which is consistent with the large MIC value of vancomycin of 512 μg/mL for VRE and the much smaller value of 4 μg/mL for *E. faecalis*. The ITC approach proposed here could be applied to screen and evaluate small molecule inhibitors of VanX or to identify drug resistant bacteria.

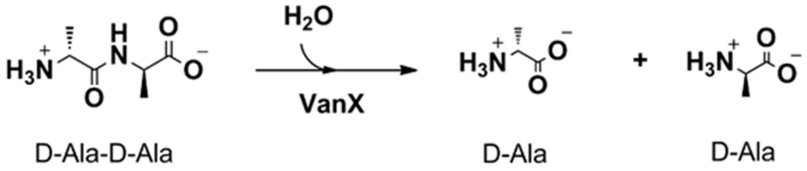
***Keywords:*** vancomycin; VanX; continuous assay; inhibition; isothermal titration calorimetry

## 1. Introduction

Vancomycin, a glycopeptide antibiotic, is widely used for the treatment of gram-positive

bacterial infections [1]. It inhibits the cell wall biosynthesis of gram-positive bacteria by binding specifically to cell wall peptidoglycans that terminate in D-Ala-D-Ala [2]. This blockade of the transpeptidation of uncrosslinked D-Ala-D-Ala containing cell wall components ultimately leads to osmotic lysis of bacteria [3]. However, the overuse of this drug has caused rapid emergence of vancomycin-resistant bacteria, such as vancomycin-resistant *Staphylococcus aureus* (VRSA) and vancomycin-resistant *Enterococci* (VRE) [4,5].

Vancomycin resistance in clinical VRSA and VRE strains require expression of five genes for high-level resistance, four of the gene products, VanS, VanR, VanH, and VanA, produce a modified peptidoglycan unit in which the terminal D-Ala is replaced by D-lactate [6-8]. The fifth gene product, VanX, a Zn(II)-dependent dipeptidase, hydrolyzes D-Ala-D-Ala as shown in Scheme 1, allowing the D,D-depsipeptide to accumulate and incorporate into the growing peptidoglycan termini mediated by the VanH and VanA enzymes [9]. Vancomycin binds to the modified peptidoglycan termini D-Ala-D-Lac 1000-fold less tightly than to D-Ala-D-Ala peptidoglycan due to the loss of one hydrogen bond, and unimpeded peptide chain cross-linking, thus yielding a mechanically strong cell wall and resistance to lysis and cell death in the presence of vancomycin [8,10-12]. Clearly, VanX is a potential drug target, and screening inhibitors of VanX is an effective way to combat vancomycin resistance [9,12,13].




**Scheme 1.** D-Ala-D-Ala hydrolysis catalysed by VanX

Given the enormous biomedical importance of VanX, many attempts have been made to develop assays for VanX activity in searching for effective compounds for inhibition of VanX [13,14]. The current methods for assaying VanX activity, such as ninhydrin/Cd(II)-based measurement and capillary electrophoresis (CE) [15,16], are noncontinuous and are limited because of lack of chromophoric a substrate. The ninhydrin/Cd(II)-based assay is time-consuming, while the disadvantages of capillary electrophoresis are poor reproducibility, narrow linear range and low sensitivity [17]. In addition, both assays are performed *in vitro* without the complete context of the physiological environment. Clearly, there is a general need for a straightforward and feasible approach that enables real-time activity assay and inhibition of VanX.

Isothermal titration calorimetry (ITC) is an ideal approach to measure the change in thermal power generated during enzyme catalyzed reactions. ITC is sensitive, has a fast response time, does not require any labelling chemistry, and provides kinetic parameters ($K_M$, $k_{cat}$, $K_i$) as well as thermodynamic information on the enzyme reaction [18,19]. The approach has been applied to

assess the activity and binding mode of enzyme inhibitors [19-21]. The experimental basis of ITC is to monitor the heat power produced or absorbed during a titration of one solution into another [22]. Processing of the data requires a model accounting for the observations [22]. Here, we report an ITC method for continuous assay of activity and inhibition of VanX *in vitro* as well as the VanX harbored in bacteria through using D-Ala-D-Ala as substrate.

## 2. Materials and methods.

### *2.1. Materials and reagents*

The substrate, D-Ala-D-Ala dipeptide and D-cysteine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The clinical bacterial strains *Enterococcus faecalis* and VRE were obtained from Clinical Laboratory, Xijing Hospital, Air Force Medical University (Xi'an, China), and *E. coli* BL21 (DE3) cells were purchased from Wolsen Co. Ltd (Xi'an, China). All other chemicals were analytical grade.

### *2.2. Overexpression and purification of VanX*

The overexpression and purification of VanX were carried out as previously reported [23]. The BL21 (DE3) *E. coli* cells containing the maltose-binding protein-VanX (MBP-VanX) in pIADL14 plasmid was cultured overnight in 4 × 1 liter of lysogeny broth (LB) containing 25 μg/mL kanamycin. The inocula were shaken at 30 °C until reaching an $OD_{600}$ of 1.8, and then were induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 75 min, harvested by centrifugation, and ruptured by ultrasound. The cell lysate was loaded onto an amylose affinity column and eluted with 30 mM Tris, pH 7.6, containing 10 mM maltose and 200 mM NaCl. The fractions containing MBP-VanX were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the pure protein fractions were concentrated. MBP was removed from MBP-VanX by thrombin digestion, the cleaved protein solution was further purified by a Q-Sepharose column eluted with a linear gradient of 0-500 mM NaCl in 30 mM Tris, pH 8.0 to offer the desired VanX. The purity of VanX was analyzed by SDS-PAGE and the concentration was determined using Beer's law with the extinction coefficient of 51,200 $M^{-1}$ $cm^{-1}$ at 280 nm.

### *2.3. Ninhydrin/Cd(II) assays*

The activity of VanX and its inhibition studies by D-cysteine were quantitatively assayed with D-Ala-D-Ala as substrate in 50 mM HEPES pH 8.0 at 37 °C as previously reported [12,15]. The absorbance was detected at 505 nm (Agilent UV-Vis 8453 spectrophotometer) and quantified with free amino acid as standard (Fig. S1). Kinetic experiments were carried out using 50 nM VanX with 0.1-0.5 mM D-Ala-D-Ala as a substrate. Rates were also determined in the presence of 10-90 µM inhibitor by pre-incubating the enzyme with D-cysteine for 30 min at room temperature before starting the kinetic experiments. The kinetic parameters were determined using the SigmaPlot v.12.0 by the equation for competitive inhibition Eq. (1), where $v$ is the rate of catalysis, $k_{cat}$ is the catalytic constant, $[E]_{tot}$ is the enzyme concentration, [S] is the initial substrate concentration, $K_M$ is the Michaelis constant, [I] is the inhibitor concentration, $K_i$ inhibition constant. The experiments were performed in triplicate using different enzyme preparations and average values were reported.

$$v = \frac{k_{cat}[E]_{tot}[S]}{[S]+K_M\left(1+\frac{[I]}{K_i}\right)} \qquad (1)$$

*2.4. Determination of MIC*

The minimum inhibitory concentration (MIC) of antibiotics against bacteria was determined using the broth micro-dilution method in 96-well microliter plate format [24,25]. Single colonies of VRE and *Enterococcus faecalis* in LB agar plates were transferred into 5 mL of Mueller-Hinton (MH) liquid medium. Strains were grown in MH medium to $OD_{600}$= 0.4 at 37 °C. The final concentrations of bacteria were diluted to $5\times10^5$ by Mueller Hinton Broth (MHB) and then added to different concentrations of Vancomycin ranging from 4 to 4096 µg/mL in a series of two fold dilutions. The mixture was incubated at 37 °C for 16 h. The MIC results were taken as the lowest concentration that completely inhibited visible growth. Each measurement was performed in duplicate.

*2.5. Calorimetric assays*

*2.5.1. Calorimetric assays with purified VanX*

In a single injection experiment, the substrate solution was injected into the cell to cause a continuous change in thermal power which eventually returned to baseline, representing full conversion of the substrate into product within a certain period of time. For the experiment with

purified VanX protein, 30 µL of substrate D-Ala-D-Ala (1, 2, 4, and 6 mM) in syringe was injected into the sample cell filled with 210 µL of 1.3 µM purified VanX prepared in the same buffer. The reference cell was loaded with deionized water and the experiments were set at 37 °C with a stirring speed of 750 rpm. After collecting initial heat rate measurements for 1 min (establishing a base line), the substrate solution was injected into the instrument sample cell. Heat rate (microJoules/second) was recorded as a function of time. Data were collected every 1 s until the signal returned to the baseline and the apparent enthalpy change ($\Delta H_{app}$) of the reaction was calculated by dividing the integrated heat ($Q_{total}$) by the n moles of substrates converted according to Eq. (2), where d$Q$/d$t$ is the heat rate, $V_{inj}$ is the volume of the injection, and $[S]_{t=0}$ is the substrate concentration.

$$\Delta H_{app} = \frac{\int_{t=0}^{\infty} \frac{dQ}{dt} dt}{V_{inj}[S]_{t=0}} = \frac{Q_{total}}{n} \qquad (2)$$

The reaction rate ($v$) is estimated from the decay heat curve of the substrate using Eq. (3), and instantaneous substrate concentrations at time $t$ are calculated from Eq. (4):

$$v = \frac{d[p]}{dt} = \frac{1}{V_{cell}\Delta H_{app}} \frac{dQ}{dt} \qquad (3)$$

$$[S]_t = [S]_{t=0} - \frac{\int_0^t \frac{dt}{dQ}}{V_{cell}\Delta H_{app}} \qquad (4)$$

where $V_{cell}$ is the volume of the sample cell. Data were fit to the Michaelis-Menten equation (Eq. 5) using non-linear least squares regression to get the kinetic constants $K_M$ and $k_{cat}$ for enzymatic hydrolysis of each substrate:

$$v = \frac{k_{cat}[E]_{tot}[S]}{K_M + [S]} \qquad (5)$$

The single injection experiments were also carried out in the presence of inhibitor by pre-incubating VanX enzyme with 10-80 µM D-cysteine prepared in the same buffer, and the inhibition constant of inhibitor were calculated by global fitting of the data to the general inhibition equation (Eq. 1) using Origin software. All experiments were performed in triplicate using different enzyme preparations and average values and standard deviations of $\Delta H_{app}$, $K_M$, $k_{cat}$, and $K_i$ were determined.

*2.5.2. Calorimetric assays with living bacterial cells*

Substrate hydrolysis within bacterial cells was studied by monitoring the change in thermal

power after the injection of 30 μL of 1-6 mM D-Ala-D-Ala in 30 mM Tris, pH 8.0 into the cell loaded with 210 μL bacterial cell suspensions. The bacterial strains used in this study were recombinant *E. coli*-VanX cells, *E. coli* BL21 (DE3) (negative control without VanX), *Enterococcus faecalis* and positive clinical isolate VRE (producing VanX). All cells were grown on LB agar plates. A single colony was picked from these plates and was incubated overnight in LB medium at 37 °C. The cultures of recombinant *E. coli*-VanX cells and of clinical VRE were then centrifuged and adjusted to an $OD_{600}$ of 2.0 with 30 mM Tris, pH 8.0 before cell suspensions were loaded for ITC studies. To determine the $IC_{50}$ values of inhibitor against VanX using the cell-based ITC method, we pre-incubated D-cysteine at various concentrations (0-320 μM) with *E. coli* cells expressing VanX and VRE for 30 min and then placed the solution in the sample cell (210 μL). After collecting initial heat rate measurements for 1 min (establishing a base line), 30 μL of 4 mM substrate solution was injected into the sample cell and the heat rate was recorded every 1 s. In addition, to verify the measured heat rate was only due to enzymatic hydrolysis of substrate, control experiments were performed by injecting substrate into buffer, substrate into the same amount of a reference strain and buffer into cell suspensions. All experiments were carried out at 37°C with a stirring speed of 750 rpm.

## 3. Results and discussion

### *3.1. Overexpression and purification of VanX*

VanX, a Zn(II) ion-containing dipeptidase, catalyzes hydrolysis of dipeptide D-Ala-D-Ala. As previously reported, VanX was overexpressed successfully as MBP-VanX fusion protein in *E. coli* BL21 (DE3) cells [23]. The MBP tag was removed by thrombin cleavage and the target protein VanX was separated by Q-Sepharose anion exchange column. Fig. 1 shows the results of overexpression and purification of VanX from *E. coli*. The obtained VanX protein had a purity of 95% (Lane 7).

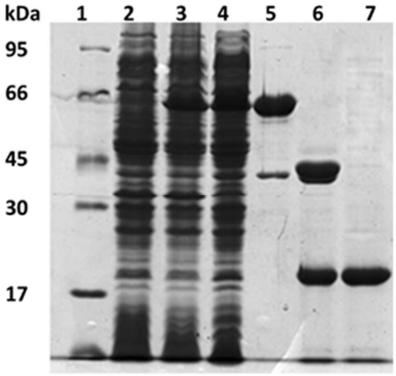



**Fig. 1.** SDS-PAGE of VanX purification. Lane 1: protein molecular weight marker. Lane 2: total cell lysate of *E. coli* BL21 cells transformed with expression vector encoding the MBP-VanX fusion protein before IPTG induction. Lane 3: total cell lysate of *E. coli* BL21 cells transformed with expression vector encoding the MBP-VanX fusion protein after 1mM IPTG induction. Lane 4: supernatant crude protein after crushing and centrifugation. Lane 5: MBP-VanX protein eluted from amylose column. Lane 6: MBP-VanX protein cleaved by thrombin. Lane 7: purified VanX protein eluted from Q-Sepharose column.

*3.2. Activity assay of VanX by ITC*

The hydrolysis of D-Ala-D-Ala by VanX (Scheme 1) involves a series of heat effects [26]. The change of thermal power (d$Q$/d$t$) generated during the reaction was monitored by Malvern MicroCal ITC200 in a multiple or single injection mode. The substrate is titrated into a solution of enzyme in the sample cell, and the d$Q$/d$t$ value is registered. Since d$Q$/d$t$ is proportional to the reaction rate, the analysis can provide the kinetic parameters ($K_M$, $k_{cat}$, $K_i$) [27,28].

ITC was employed to monitor the hydrolysis of D-Ala-D-Ala by VanX in a single injection mode. Successive titration of 30 µL of 4 mM D-Ala-D-Ala into the sample cell filled with 1.3 µM VanX and injection of substrate to buffer was performed as a control. The typical thermograms are shown in Fig. 2A. A decrease of thermal power (d$Q$/d$t$) following injection of the substrate is observed, indicating that the hydrolysis is an exothermic reaction. The thermal power generated (d$Q$/d$t$) reached the maximum at ~140 s, and heat rate returned to baseline within 220 s, suggesting that the substrate was hydrolysed completely. The power (d$Q$/d$t$) at each substrate concentration, obtained by measuring baseline displacement, was converted to enzyme turnover rates according to Eq. (3). The reaction rate data were then analyzed by the Origin program provided by Malvern company and were further fitted to the Michaelis-Menten equation (Eq. 5) using non-linear least squares regression (Fig. 2B) to give the kinetic constants $K_M$ (0.10 mM) and $k_{cat}$ (11.5 $s^{-1}$) (Table 1).

To examine the reliability of the ITC approach, the ninhydrin/Cd(II)-based assay was also

employed to evaluate the substrate hydrolysis as previously reported [15]. The resulting $K_M$ and $k_{cat}$ values (0.14 mM and 43.8 $s^{-1}$) are in agreement with the data from ITC assay (considering the difference in the two sets of assays and that different enzyme concentrations were used). $K_M$ and $k_{cat}$ are also consistent with the data (0.11 mM and 46 $s^{-1}$) reported by Anissimova *et al*. [17], indicating that the ITC method is a viable approach for monitoring VanX activity.

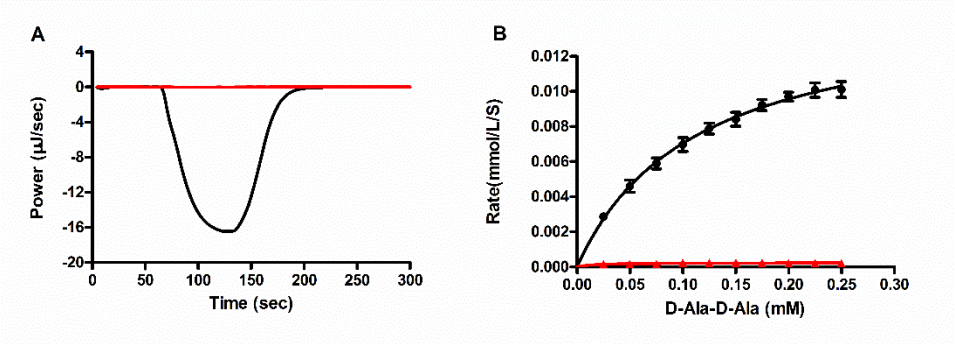



**Fig. 2.** Enzyme kinetics of VanX catalyzed D-Ala-D-Ala hydrolysis using ITC assay in a single injection method. (A) Raw thermal power observed in the calorimetric assay, performed at 37 °C with 30 µL injections of 4 mM D-Ala-D-Ala in the syringe into 1.3 µM VanX (black) and buffer (red) in the sample cell. (B) Global fitting of VanX enzymatic activity using data from the single injection ITC assay. Baseline displacement of the thermal power after injection of the substrate was converted to reaction rate and was plotted according to the Michaelis-Menten equation to give the values of $K_M$ and $k_{cat}$.

*3.3 Inhibition assay of VanX by ITC*

To investigate whether the method could be used in inhibition assay of VanX, we assessed D-cysteine, a previously reported inhibitor of VanX [13], in the single injection mode of ITC. The inhibition of D-Ala-D-Ala hydrolysis with VanX by D-cysteine is shown in Fig. 3A. It clearly shows that the compound inhibits VanX effectively, a high concentration of inhibitor leads to a low initial hydrolysis rate of D-Ala-D-Ala. Through fitting the initial velocity versus substrate concentration at each inhibitor concentration, we determined the $K_i$ value of D-cysteine to be 18.8 µM, which is consistent with the value (16 µM) reported [13]. Also, using the ninhydrin/Cd(II)-based assay (Fig. 3B), we determined the $K_i$ value to be 23.2 µM, which is in agreement with the data from the ITC assay. These results suggest that the ITC method could be used for screening inhibitors of VanX.

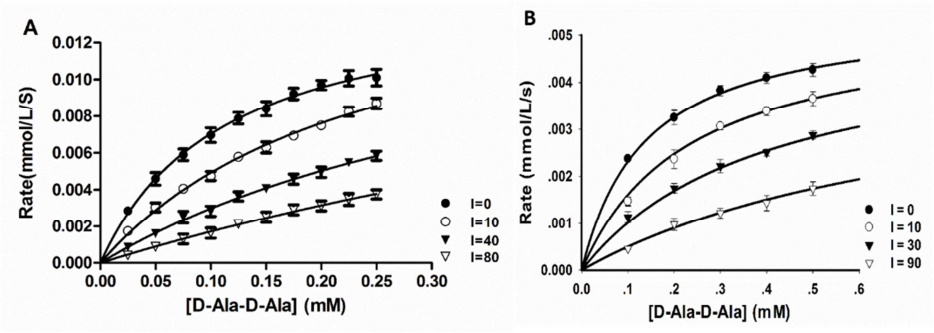



**Fig. 3.** Inhibition of D-Ala-D-Ala hydrolysis with VanX by D-cysteine. (A) Reaction rate of hydrolysis substrate at different substrate concentrations in the absence and presence of D-cysteine calculated from the single injection ITC assay. The data was fitted according to the Michaelis-Menten equation to obtain the value of $K_i$. The baseline controls without VanX were subtracted from the experimental data to offer the overlaid heat flow curves. (B) Michaelis-Menten plots to determine the kinetic parameters of D-cysteine using the ninhydrin/Cd(II)-based method.

**Table 1. The determined kinetic data of VanX hydrolyzing D-Ala-D-Ala and inhibition of VanX by D-cysteine through ITC and Ninhydrin/Cd(II)-based method**

| Method | $K_M$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}/mM^{-1}$) | $K_i$ (μM) |
|---|---|---|---|---|
| ITC | 0.10 ± 0.01 | 11.5 ± 0.3 | 115.0 | 18.8 ± 0.2 |
| Ninhydrin/Cd(II) | 0.14 ± 0.03 | 43.8 ± 0.2 | 312.9 | 23.2 ± 0.3 |

*3.4 Inhibition assays of VanX in living bacterial cells*

Given the enormous biomedical importance of VanX, it is necessary to develop a straightforward and feasible approach to monitor small molecules such as drugs as inhibitors or substrates of VanX in living cells. In a single injection mode of ITC, a typical heat rate curve of VanX catalyzed hydrolysis of D-Ala-D-Ala is shown in Fig. S2. As the concentration of the substrate decreased, the heat rate signal returns gradually to baseline, which indicates the end of the reaction.

The single injection mode of ITC was employed to monitor hydrolysis of D-Ala-D-Ala by the VanX enzyme and *E. coli* BL21 (DE3) that produce VanX, while the *E. coli* cells without VanX were monitored as a control. The progress of D-Ala-D-Ala hydrolysis monitored by ITC is showed in Fig. 4. It clearly shows that the dipeptide at different concentration is quickly hydrolyzed when it is treated with the purified VanX enzyme (Fig. 4A), and an increase of substrate concentration results in an increase of d*Q*/d*t* values. Under the *in vivo* test conditions employed (6 mM D-Ala-D-Ala and a suspension of *E. coli* cells with an optical density at 600 nm ($OD_{600}$) of 2.0 in 30

mM Tris-HCl, pH 8.0), D-Ala-D-Ala is quite stable, if the *E. coli* do not express VanX (Fig. 4B). The weak thermal power (< 2.1 μJ/s) observed in response to various concentrations of D-Ala-D-Ala is due to the heat of dilution and/or intrinsic bacteria metabolic heat [29,30]. However, the D-Ala-D-Ala is hydrolyzed in the presence of *E. coli* producing VanX (Fig. 4C).

To test whether the VanX enzyme remained inside the cells or leaked into the medium during the ITC experiment, the *E. coli* cells expressing VanX and treated with D-Ala-D-Ala were removed by centrifugation and the resulting cell-free supernatant was supplemented with fresh dipeptide. No significant thermal power caused by dipeptide hydrolysis was observed in the cell-free supernatant (Fig. S3), indicating that VanX enzyme had not leaked from *E. coli* cells into the medium, and confirming that the thermal power was contributed by VanX expressed in *E.coli* (Fig. 4C). The thermograms of substrate hydrolysis by the purified VanX enzyme and by *E. coli* producing VanX are extremely similar, both reaching maximal hydrolysis rates at ~140 s. However, the maximum thermal power reached during the conversion of 6 mM D-Ala-D-Ala with bacteria is ~18.8 μJ/s, which is significantly smaller than ~20.9 μJ/s for purified VanX. This may be due to the different amount of VanX expressed in bacteria as compared to that used with purified enzyme.

To assess the bacteria metabolic heat and/or dilution heat, titration of 30 μL of D-Ala-D-Ala (4 mM) into the VanX free *E. coli* cells and 30 μL Tris buffer into the *E. coli* cells with and without VanX with different $OD_{600}$ values, and the results (Fig.S4 and S5) show that the thermal power changed very little from -0.2 to -1.0 μJ/sec with the increase of $OD_{600}$ value from 0.5 to 6, suggesting that the residual heat is mainly from the dilution of enzyme substrate or buffer during the assays, because the metabolic heat derived from cell growth often took place over a much longer period of time (hours to days) [31]. To further check if the viability of the *E. coli* cells expressing VanX was affected by the ITC procedure, a plating colony test was carried out before and after the ITC experiment. The results showed that ITC experiments for one hour did not lead to any change in cell viability (Fig. S6).

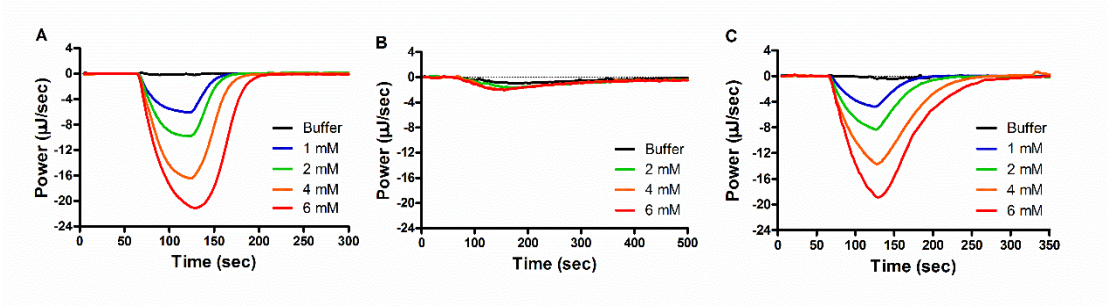



**Fig. 4.** Overlaid heat flow curves of D-Ala-D-Ala hydrolysis by purified VanX (A), *E. coli* BL21 cells (B) and *E. coli* BL21 expressing VanX (C), ($OD_{600}$ = 2). All samples were prepared in 30 mM Tris-HCl, pH 8.0. The heat rate during hydrolysis of D-Ala-D-Ala (1-6 mM in syringe) was measured every 1 s.

To verify whether the cell-based ITC method could be applied to clinical strain assays, we examined the degradation of D-Ala-D-Ala by two clinical isolates: VRE which expresses VanX, and *Enterococcus faecalis* which does not express VanX. The progress of D-Ala-D-Ala hydrolysis monitored by ITC is shown in Fig. 5. The substrate at different concentrations was quickly hydrolysed by VRE, increased substrate concentration resulted in increased d$Q$/d$t$ values, but not by *Enterococcus faecalis*. Given the consistency of total heat ($Q_{total}$) obtained by ITC and the susceptibility of vancomycin [32], we determined the minimum inhibitory concentration (MIC) of vancomycin against above two clinical isolates. The MIC values of VRE and *E. faecalis* are 512 and 4 μg/mL, respectively (Table 2), which are consistent with the $Q_{total}$ values (1.26 mJ for VRE and 0.031 mJ for *E. faecalis* at 4 mM), suggesting that the ITC method can be used to screen clinical strains for VanX by monitoring the heat generated during the hydrolysis of substrate.

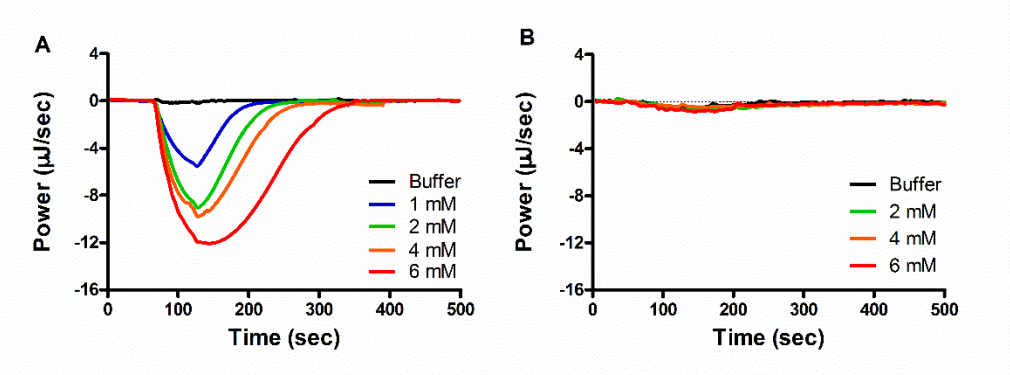



**Fig. 5.** Overlaid heat rate curves of D-Ala-D-Ala hydrolysis by VRE (A) and *Enterococcus faecalis* (B) ($OD_{600}$ = 2). All samples were prepared in 30 mM Tris-HCl, pH 8.0. The heat rate during hydrolysis of D-Ala-D-Ala (1-6 mM in syringe) was measured every 1 s.

**Table 2. MIC and total heat of vancomycin against clinical isolates VRE expressing VanX and**

***Enterococcus faecalis***

| Bacteria | MIC (μg/mL) | Susceptibility | $Q_{total}$ (mJ) |
|---|---|---|---|
| VRE (VanX) | 512 | resistance | 1.26 ± 0.07 |
| *Enterococcus faecalis* | 4 | sensitive | 0.031 ± 0.002 |

Further, the ITC approach was employed to assess the inhibition action of D-cysteine to *E. coli* and VRE cells that express VanX. 30 μL of 4 mM D-Ala-D-Ala was injected into the sample cell filled with 210 μL of pre-incubated inhibitor at different concentrations with VanX protein solution at a concentration of 1.30 μM. The same experiments were performed with *E. coli* cells expressing VanX or VRE at an $OD_{600}$ of 2.0. To rule out the dilution effects, the control experiments were performed with the test buffer lacking VanX, *E. coli* and *Enterococcus faecalis* cells that do not express VanX. ITC data monitoring inhibition (corrected for the almost negligible control data, Fig. S7A, S7B, and S7C) is shown in Fig. 6. The higher the inhibitor concentrations, the lower the hydrolysis rates of substrate (Fig. 6A, 6B, and 6C). In the absence of inhibitor, the $(dQ/dt)_{max}$ value corresponding to the rate of uninhibited enzyme-catalyzed D-Ala-D-Ala hydrolysis was registered as 100% enzyme activity [33]. We determined the fifty percent inhibitory concentration ($IC_{50}$) values of D-cysteine to be 25.1, 29.8 and 28.6 μM, respectively (Fig. 6D, 6E and 6F), which are in agreement with $K_i$ values (23.2) that we determined and the data (16 μM) reported from ninhydrin/Cd(II)-based assay *in vitro* [13], indicating that this approach could be used for assaying inhibition of VanX *in vitro* and *in vivo*.

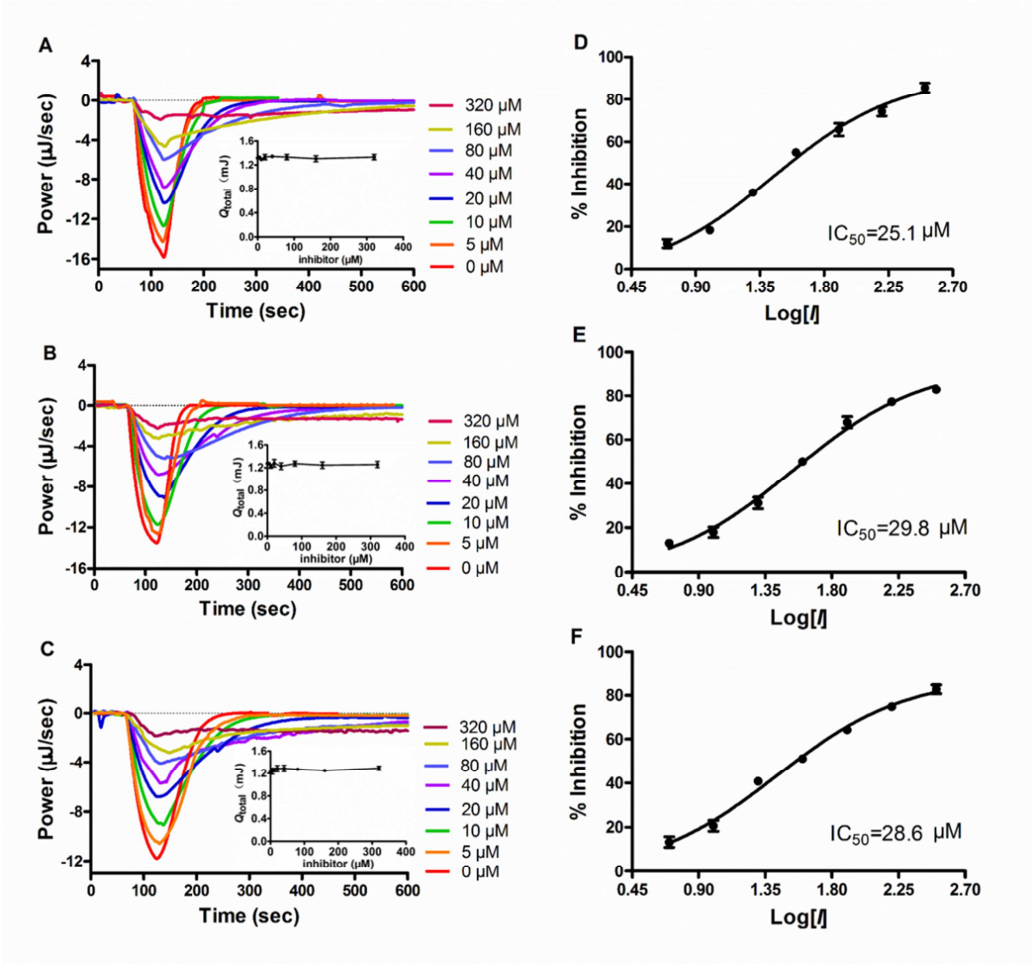



**Fig. 6.** Overlaid heat rate curves of D-Ala-D-Ala hydrolysis by purified VanX enzyme (A), *E. coli* cells expressing VanX (B) and VRE (C) in the presence of D-cysteine at various inhibitor concentrations, and $IC_{50}$ measurements for D-cysteine to purified VanX enzyme (D), *E. coli* cells expressing VanX (E) and VRE (F). The insets in A-C show that total heat $Q_{total}$ calculated by integrating the different power curves obtained with inhibitor at various concentrations is constant, as expected. The baseline controls without VanX expression (Fig. S6) were subtracted from the experimental data to offer the overlaid heat flow curves. 30 μL of 4 mM substrate was injected to 210 μL of VanX solution at a concentration of 1.30 μM or *E. coli* cells expressing VanX or VRE at an $OD_{600}$ of 2.0.

## 4. Conclusions

Based on the heat absorbed or released during the specific reaction of an enzyme with its substrate, we report a simple and straightforward ITC method using D-Ala-D-Ala as substrate for continuous real-time activity and inhibition assay of VanX enzyme, VanX harboured in *E. coli* cells, and clinical isolate VRE expressing VanX. This method allowed the determination of kinetic parameters of VanX hydrolyzing the dipeptide *in vitro* and determination of $IC_{50}$ values of D-cysteine inhibiting VanX harboured in clinical isolate. The cell-based ITC method proposed here

could be applied to screen and evaluate small molecule inhibitors of VanX in whole cells or to identify drug resistant bacteria.

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**Abbreviations**

ITC, isothermal titration calorimetry; VRSA, vancomycin-resistant *Staphylococcus aureus*; VRE vancomycin-resistant *Enterococci*; MBP, maltose-binding protein; LB, lysogeny broth; *E. coli*, *Escherichia coli*; $IC_{50}$, fifty percent inhibitory concentrations; MIC, minimal inhibitory concentration.

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Research highlights

- Continuous assay of VanX activity by isothermal titration calorimetry (ITC) is described.
- The inhibition constant of VanX enzyme inhibitor, D-Ala-D-Ala, is given.
- The method can be applied to evaluate the activity of VanX inside living bacterial.