Chapter 19

Structural Comparison and Simulation of Pneumococcal Peptidoglycan Hydrolase LytB

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Abstract

Three-dimensional structural determination combined with comprehensive comparisons with the homologs is a straightforward strategy to decipher the molecular function of an enzyme. However, in many cases it's difficult to obtain the complex structure with the substrate/ligand. Structure-based molecular simulation provides an alternative solution to predict the binding pattern of a substrate/ligand to the enzyme. The *Streptococcus pneumoniae* LytB is a peptidoglycan hydrolase that cleaves the glycosidic bond and therefore involves the cell division; however, the details of catalytic mechanism remain unknown. Based on the crystal structure of the catalytic domain of LytB (termed $LytB_{CAT}$), we describe here how to assign the molecular functions of three $LytB_{CAT}$ modules: SH3b, WW, and GH73, using structural comparisons. Moreover, we dock a putative tetrasaccharide-pentapeptide substrate of peptidoglycan onto $LytB_{CAT}$ to provide the details of substrate binding pattern. The tetrasaccharide-pentapeptide is well accommodated in a T-shaped substrate binding pocket formed by the three modules. The conclusions deduced from structural comparison and simulation are further proved by the hydrolytic activity assays in combination with site-directed mutagenesis.

Key words *Streptococcus pneumoniae*, Peptidoglycan, Peptidoglycan hydrolase, LytB, Structural comparison, Simulation, Hydrolytic activity assay

1 Introduction

Peptidoglycan (PG), also known as murein, is the major and specific component of bacterial cell wall. It withstands cell turgor in order to maintain cell shape and preserve cell integrity [1]. PG comprises alternating β(1,4)-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues, attached by cross-linked short peptides to form a three-dimensional structure [1, 2]. Subtle "destruction" or remodeling of PG is crucial for bacterial cell growth and division [3]. It requires highly diverse group of hydrolases to cleave different covalent bonds of PG [4]. In the past decades, several PG hydrolases had been identified in human

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pathogen *Streptococcus pneumoniae*, such as autolysin LytA [5], lysozyme LytC [6], and so on. In 1999, LytB was initially characterized as a PG hydrolase for the reason that the *lytB* knockout pneumococci were deficient in cell separation and formed long-chains [7]. Subsequently, García et al. found that the purified recombinant LytB is capable of dispersing the long-chains of *lytB* knockout pneumococci, indicating that LytB possesses a glucosaminidase activity to cleave the β(1,4)-linked glycosidic bond between NAG and NAM [8]. Thus, LytB may play an indispensable role in cell division. Recently, we reported the crystal structure of the catalytic domain of LytB (residues Lys375-Asp658, termed $LytB_{CAT}$) [9].

As we know, the similarity analysis of protein structure is a vital step in understanding protein's function. Here, we divide $LytB_{CAT}$ into three distinct modules: a C-terminal α-helix module and two all-β modules, and then identify their function by comparing the structure of each module with the known structures, respectively. According to primary sequence analysis, the C-terminal α-helix module (residues Gly494-Asp658) is classified into the glycoside hydrolase family 73 (GH73) [10]. Then we superimpose this module onto the only two known structures of GH73: the surface associated autolysin Auto from *Listeria monocytogenes* (PDB code 3fi7) [11] and the flagellar protein FlgJ from *Sphingomonas* sp. (PDB code 2zyc) [12], using SUPERPOSE [13] as a part of the CCP4i [14] on the basis of secondary structure matching (SSM) algorithm. The results suggest that this α-helix module possesses a GH73 fold and functions as a catalytic module, with Glu564 as the catalytic residue. Concerning the first all-β module (residues Asn385-Ser450), we use Dali server [15] to search homologous structures, which are in turn applied to structural superpositions against the input structure. The results indicate that the first all-β module may resemble SH3b domain and contribute to PG recognition. However, the Dali search against the second all-β module (residues Lys451-Asp493) yields no significant homologs. Instead, after searching against the Structural Classification of Proteins (SCOP) database, it is identified as a WW domain-like fold which probably binds to the carbohydrate moiety of PG, and can be well superimposed onto the chitin binding domain (ChBD) of *Serratia marcescens* chitinase ChiB (PDB code 1e15) [16]. Hence, $LytB_{CAT}$ is divided into three structurally independent modules: $LytB_{SH3b}$, $LytB_{WW}$, and $LytB_{GH73}$.

Though LytB has been proved to cleave the NAG-(β-1,4)-NAM glycosidic bond of PG at the septum to separate two daughter cells [8], its bona fide physiological substrate remains undefined. Due to the commercial unavailability of the complex fragments of PG, we choose to simulate a PG fragment that mimics the physiological substrate, to provide the details of substrate binding pattern of $LytB_{CAT}$. Molecular simulation is a computational procedure

that attempts to predict noncovalent binding of a macromolecule (receptor) and a small molecule (substrate/ligand). Among various tools of simulation, AutoDock has been proved to be able to effectively and accurately predict the conformations and binding affinity of a substrate/ligand towards the target macromolecule [17]. AutoDock Vina automatically calculates the grid maps and clusters the results in a transparent way [18]. It speeds up the gradient optimization by using a simpler scoring function and therefore significantly improves the accuracy of the binding mode predictions. A T-shaped substrate binding pocket can be found from the electrostatistic potential diagram of $LytB_{CAT}$, which is reminiscent of a PG fragment: *t*etra*s*accharide-*p*enta*p*eptide NAM-NAG-NAM (-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala)-NAG (TSPP) as the putative substrate. Then we generate the atomic coordinates of TSPP using PRODRG Server [19] and dock it onto $LytB_{CAT}$ using AutoDock Vina [18]. The final simulated model suggests that the tetrasaccharide moiety of TSPP is accommodated in the groove of $LytB_{GH73}$, whereas the pentapeptide moiety stretches into the cleft between $LytB_{SH3b}$ and $LytB_{WW}$.

In order to prove the above results of structural comparisons and simulation, we test the contribution of each module of $LytB_{CAT}$ to the hydrolytic activity of $LytB_{CAT}$. LytB hydrolyzes the wild-type PG at a much lower velocity compared to the PG purified from the *lytB* knockout strain (Δ*lytB* PG) [9], in agreement with that LytB probably prefers immature PG [8]. Thus Δ*lytB* PG is applied to all hydrolytic activity assays. We label Δ*lytB* PG with Remazol Brilliant Blue (RBB), and then incubate it with different versions of recombinant $LytB_{CAT}$ protein ($LytB_{CAT}$, $LytB_{WW\text{-}GH73}$, $LytB_{GH73}$, $LytB^{E564Q}$) at 37 °C for 10 h. After terminating the reaction, the activity of each protein sample is calculated by detecting the amount of RBB-labeled Δ*lytB* PG released to the supernatant upon hydrolysis. The results show that Glu564 plays a crucial role in hydrolysis, and none of the three modules is dispensable for the activity of $LytB_{CAT}$. The results indicate the reliability of structural comparisons and simulation.

2 Materials

2.1 The Atomic Coordinates

The atomic coordinates used in structural comparisons are listed in Table 1 (*see* **Note 1**).

2.2 Websites and Programs

1. UniProt: Universal Protein Resource, http://www.uniprot.org/. It provides the scientific community with a comprehensive, high-quality, and freely accessible resource of protein sequences and functional information.

Table 1
The atomic coordinates used in structural comparisons

	PDB code	Bacterial species	Description
$LytB_{CAT}$.pdb	4q2w	*S. pneumoniae*	The catalytic domain of LytB
3fi7.pdb	3fi7	*L. monocytogenes*	The GH73 domain of the surface associated autolysin Auto
2zyc.pdb	2zyc	*Sphingomonas* sp.	The GH73 domain of the flagellar protein FlgJ
2hbw.pdb	2hbw	*A. variabilis*	The SH3b domain of the γ-D-glutamyl-L-diamino acid endopeptidase AvPCP
1r77.pdb	1r77	*S. capitis*	The SH3b domain of peptidoglycan hydrolase ALE-1
1e15.pdb	1e15	*S. marcescens*	The chitin binding domain of chitinase ChiB

2. PyMOL: http://www.pymol.org/. A user-sponsored molecular visualization system on an open-source foundation.
3. CCP4: A world-leading, integrated suite of programs that allows researchers to determine macromolecular structures by X-ray crystallography, and other biophysical techniques [14] (*see* **Note 2**).
4. Dali server: http://ekhidna.biocenter.helsinki.fi/dali_server/. A network service for comparing protein structures in 3D, comparing the submitted coordinates of a query protein structure against those in the PDB [15].
5. SCOP: Structural Classification of Proteins, http://scop.mrc-lmb.cam.ac.uk/scop/. It aims to provide a detailed and comprehensive description of the structural and evolutionary relationships between all proteins whose structure is known.
6. PRODRG server: http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg. It takes a description of a small molecule and from it generates a variety of topologies for use with GROMACS, Autodock, and other programs, as well as energy-minimized coordinates in a variety of formats [19].
7. Autodock: A suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. AutoDock Tools (ADT) 1.5.4 [18] and AutoDock Vina software (version 1.0) [20] are used.
8. GraphPad: A powerful combination of biostatistics, curve fitting (nonlinear regression) and scientific graphing.

2.3 Hydrolytic Activity Assays

1. Recombinant proteins: The wild-type $LytB_{CAT}$ protein and different mutated versions of $LytB_{CAT}$ protein ($LytB_{WW\text{-}GH73}$, $LytB_{GH73}$, $LytB^{E564Q}$) are constructed and purified according to a previous report [9].
2. Δ*lytB* PG is purified from the *lytB* knockout TIGR4 strain as previously reported [21]. The chromosomal *lytB* knockout strain from *S. pneumoniae* wild-type TIGR4 strain is generated by allelic replacement according to Bricker and Camilli [22].
3. 20 mM Remazol Brilliant Blue (RBB; Sigma): The RBB powder is dissolved in 0.25 M NaOH (*see* **Note 3**).
4. 0.25 M HCl: diluted from the 11 M HCl with double-distilled water (ddH_2O) to neutralize the reaction.
5. Reaction buffer: 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0
6. Centrifuge (HITACHI, Japan).
7. DU800 spectrophotometer (Beckman Coulter, Fullerton, CA).

3 Methods

After careful structural analyses, the overall structure of $LytB_{CAT}$ is divided into three distinct modules packing against each other: two all-β modules (residues Asn385-Ser450 and Lys451-Asp493, respectively) followed by a C-terminal α-helix module (residues Gly494-Asp658).

3.1 Structural Comparison of the C-Terminal α-Helix Module

1. Search "LytB in *S. pneumoniae*" in the UniProt website to collect the related information of LytB.
2. Based on the primary sequence analysis of Pfam database showed in UniProt, the C-terminal α-helix module of $LytB_{CAT}$ is defined as a glucosaminidase (PF01832), belonging to the glycoside hydrolase family 73 (GH73). GH73 is a family of glycoside hydrolases that include peptidoglycan hydrolases of endo-β-N-acetylglucosaminidase specificity. Therefore, the C-terminal α-helix module is assigned to the catalytic module of $LytB_{CAT}$. To date, only the structures of two members in this family: the surface associated autolysin Auto from *L. monocytogenes* [11] and the flagellar protein FlgJ from *Sphingomonas* sp. [12], had been solved according to the summary of the Pfam database [10] (*see* **Note 4**).
3. Open the atomic coordinates of $LytB_{CAT}$ (PDB code 4q2w, $LytB_{CAT}$.pdb) by PyMOL, and show the protein sequence. Select residues Gly120-Asp284 (corresponding to Gly494 to Asp658 in the full-length protein sequence) and then save it as $LytB_{GH73}$.pdb.

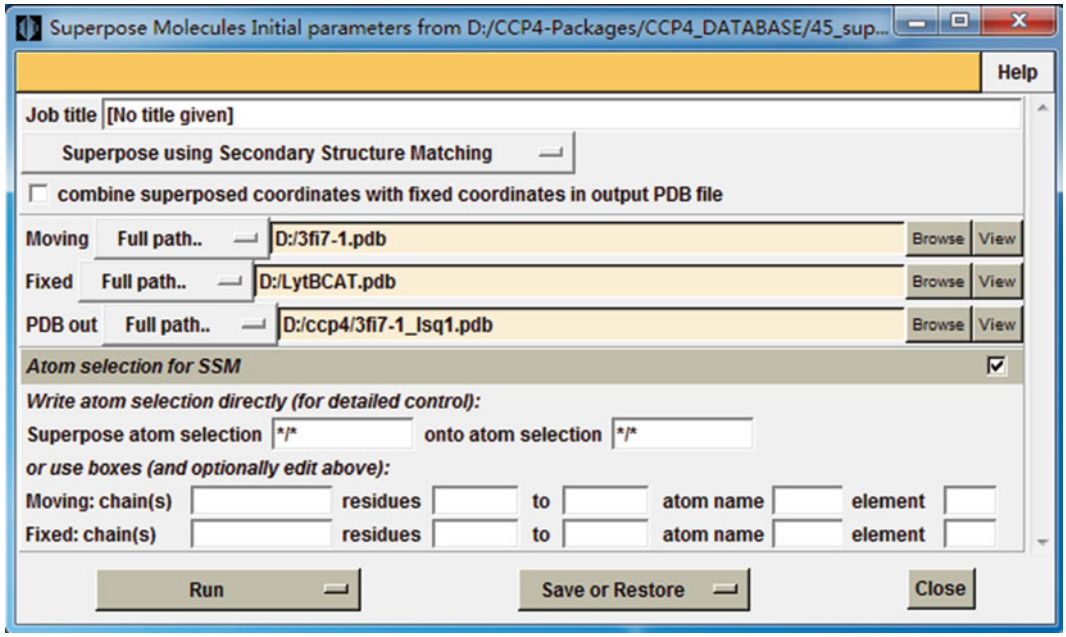

Fig. 1 The Superpose interface in CCP4i program suite 6.4.0 of superimposing $LytB_{CAT}$ against the GH73 domain of Auto (3fi7.pdb)

4. Superimpose the $LytB_{GH73}$.pdb with the GH73 domains of Auto (PDB code 3fi7) and FlgJ (PDB code 2zyc) by SUPERPOSE [13] as a part of the CCP4i [14] program suite, respectively. The atomic coordinates of the GH73 domains of Auto and FlgJ are termed 3fi7.pdb and 2zyc.pdb, respectively (*see* **Note 5**).
5. Open Superpose interface in CCP4i program suite 6.4.0, select "Superpose using Secondary Structure Matching". Fixed $LytB_{GH73}$.pdb, moving 3fi7.pdb or 2zyc.pdb, and then run the program (Fig. 1) (*see* **Note 6**).
6. View and analyze the output PDBs. Open the output 3fi7_lsq1.pdb or 2zyc_lsq1.pdb using PyMOL and then open $LytB_{GH73}$.pdb in the same window. The $LytB_{GH73}$.pdb will automatically superimposed onto the 3fi7_lsq1.pdb or 2zyc_lsq1.pdb. The results show that $LytB_{GH73}$ can be well superimposed with the GH73 domains of Auto and FlgJ, with a root mean square deviation (RMSD) of 2.12 and 1.96 Å over 94 and 86 Cα atoms, respectively. Furthermore, $LytB_{GH73}$ possesses a similar active site compared with the two GH73 domains, especially the catalytic residue. Altogether, it indicates that the C-terminal α-helix module of $LytB_{CAT}$ possesses a GH73 fold and functions as a catalytic module, with Glu564 as the catalytic residue.

3.2 Homology Search of the Two All-β Modules

1. In PyMOL, open $LytB_{CAT}$.pdb and show its protein sequence. The two all-β modules: residues Asn385-Ser450 and Lys451-Asp493 numbering in the full-length LytB, correspond to Asn11-Ser76 and Lys77-Asp119 in the $LytB_{CAT}$.pdb file. Select the residues of each all-β module, then save as β1.pdb and β2.pdb, respectively (*see* **Note 7**).
2. The Pfam database cannot classify the two all-β modules into any known family on the basis of primary sequence. Thus Dali server is chosen as an alternative tool for comparisons with structures deposited in the PDB database, to identify to which family the two all-β modules may belong and their probable function.
3. In the Dali server website, upload the atomic coordinate file (β1.pdb or β2.pdb), enter your own email address and then press "submit" (*see* **Note 8**).
4. The output is normally received in an hour or several hours later. Carefully check all hits and summarize (*see* **Note 9**).
5. In the output of the first all-β module, most proteins with a Z-score of ≥5.1 contain SH3b domain, which were predicted or hypothetical bacterial cell wall hydrolases. The first all-β module may resemble SH3b domain, thus termed $LytB_{SH3b}$. The only two well-characterized hits are the SH3b domain of the γ-D-glutamyl-L-diamino acid endopeptidase AvPCP from *A. variabilis* [23] and that of *S. capitis* peptidoglycan hydrolase ALE-1 [24], both of which appear to contribute to substrate binding.
6. Superimpose $LytB_{SH3b}$ against the SH3b domain of AvPCP (PDB code 2hbw) or ALE-1 (PDB code 1r77). The atomic coordinates of the SH3b domains of AvPCP and ALE-1 are termed 2hbw.pdb and 1r77.pdb, respectively. Run the superposition as **step 5** and **step 6** in Subheading 3.1. $LytB_{SH3b}$ shares a fold quite similar to the SH3b domains of AvPCP and ALE-1, with an RMSD of 2.5 and 2.1 Å over 60 and 58 Cα atoms, respectively. It suggests that $LytB_{SH3b}$ might contribute to substrate binding.
7. However, concerning the second all-β module, no significant results have been found (*see* **Note 10**).
8. Alternatively, process a homology search for the second all-β module in SCOP. Choose the ASTRAL database (SCOP domain sequences and pdb-style coordinate files) in the "Access methods" item, and analyze all structures in the "all beta proteins" class. The fold No. 70, called WW domain-like, is the only fold that consists of a 3-stranded meander beta-sheet similar to the second all-β module, which is in consequence termed $LytB_{WW}$. Superimpose each known structure of WW domain-like fold against $LytB_{WW}$ as **step 5** and **step 6** in

Subheading 3.1. Only the chitin binding domain (ChBD) of *S. marcescens* chitinase ChiB (PDB code 1e15, 1e15.pdb) [16] can be well superimposed onto $LytB_{WW}$, with an RMSD of 1.6 Å over 26 Cα atoms. The ChBD belongs to the carbohydrate binding domain superfamily in WW domain-like fold, indicating that $LytB_{WW}$ may also contribute to binding carbohydrate substrates (*see* **Notes 11** and **12**).

3.3 Simulation of $LytB_{CAT}$ Against the Putative Substrate

1. Analyze $LytB_{CAT}$.pdb to check whether it exists a possible substrate binding pocket on the surface of $LytB_{CAT}$. Open $LytB_{CAT}$.pdb with PyMOL and generate its "protein contact potential (local)" in vacuum electrostatistic item. A T-shaped pocket can be clearly seen from the electrostatistic potential diagram, which is most likely the putative substrate binding pocket. The T-shaped pocket comprises a groove through the catalytic module $LytB_{GH73}$, in addition to a cleft between $LytB_{SH3b}$ and $LytB_{WW}$.
2. Considering the reported structure of PG [1], this T-shaped substrate binding pocket is clearly reminiscent of an extended repetitive unit of PG, namely the *t*etra*s*accharide-*p*enta*p*eptide NAM-NAG-NAM(-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala)-NAG (termed TSPP). Simulating TSPP, which mimics the physiological substrate, onto $LytB_{CAT}$ may provide the details of substrate binding pattern.
3. Generate the atomic coordinates of TSPP by the GlycoBioChem PRODRG2 Server [19]. Click "Get started…" in the bottom of the PRODRG website to open the compound submission window. Firstly, submit your email address to the server to get a valid token before using (*see* **Note 13**).
4. Secondly, paste the obtained token and click "Draw the molecule with JME". JME is a molecular editor tool for structure input and editing. In the new opened window, draw the chemical formula of TSPP. However, do not close the original window. After finish drawing, click "transfer to PRODRG window", and the automatically generated coordinate data will be displayed in the compound submission window (*see* **Note 14**).
5. Finally, run PRODRG. In the result page, download the generated coordinate file in pdb format. Thus the coordinate file of TSPP is termed TSPP.pdb (*see* **Note 15**).
6. Open $LytB_{CAT}$.pdb using AutoDock Tools (ADT) 1.5.4 [20]. Then edit it to add polar hydrogen atoms and save as a PDBQT format in the Grid item. Select a grid box with dimensions of $40 \times 45 \times 50$ points around the active site to accommodate TSPP. Write down the number of points in *x*,*y*,*z* dimensions and numerical values of Center Grid Box in *x*,*y*,*z* (*see* **Note 16**).

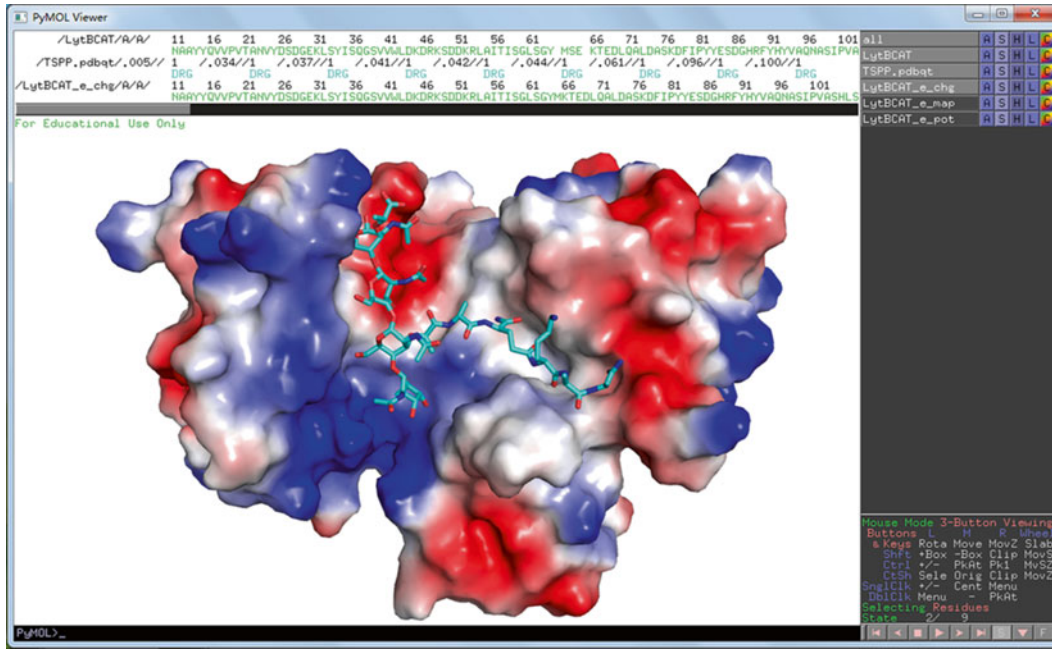

Fig. 2 The PyMOL interface that shows TSPP onto $LytB_{CAT}$

7. Delete the opened $LytB_{CAT}$.pdb and input the TSPP.pdb. In the torsion tree item, all single-bonds within the TSPP are set to allow rotation. Afterwards, convert the TSPP.pdb from a PDB format to a PDBQT format (*see* **Note 17**).
8. Build a new txt file that includes the names of receptor ($LytB_{CAT}$.pdbqt) and ligand (TSPP.pdbqt), the six parameters of the grid box and the exhaustiveness parameter (*see* **Note 18**).
9. Invoke the AutoDock Vina.exe and the above built txt file. Now the Vina will run to simulate the TSPP onto $LytB_{CAT}$ (*see* **Note 19**).
10. Open the output PDBQT file and the original $LytB_{CAT}$.pdb file using PyMOL. Analyze the given conformations and orientations of TSPP at the active site of $LytB_{CAT}$ one by one, and select the most rational one as the final model (Fig. 2). The simulated model showed that the tetrasaccharide moiety is accommodated in the groove of $LytB_{GH73}$, whereas the pentapeptide moiety stretches into the cleft between $LytB_{SH3b}$ and $LytB_{WW}$ (*see* **Note 20**).

3.4 Hydrolytic Activity Assays

1. Label the purified Δ*lytB* PG with RBB: Incubate Δ*lytB* PG with 20 mM RBB in 0.25 M NaOH at 37 °C overnight, and subsequently neutralize the reaction system with 0.25 M HCl. Then centrifuge the mixture at 21,000 × *g* for 20 min at 20 °C

to collect the precipitate. Wash the RBB-labeled Δ*lytB* PG six times with ddH_2O to remove the free RBB, and then weigh it after lyophilizing (*see* **Note 21**).

2. Dissolve the lyophilized RBB-labeled Δ*lytB* PG and dilute different versions of protein ($LytB_{CAT}$, $LytB_{WW\text{-}GH73}$, $LytB_{GH73}$, $LytB^{E564Q}$) with the reaction buffer.
3. Mix 10 μM purified protein and 1 mg/mL RBB-labeled Δ*lytB* PG in a 150 μL system and react at 37 °C for 10 h (*see* **Note 22**).
4. Heat the mixture at 95 °C for 5 min to terminate the reaction.
5. Afterwards, centrifuge the mixture at 130,000 × *g* for 20 min at 20 °C to remove the remaining insoluble PG that has not been hydrolyzed (*see* **Note 23**).
6. Apply the soluble RBB-labeled PG fragments, which are released to the supernatant upon hydrolysis, to a DU800 spectrophotometer to measure the optical density at 595 nm (*see* **Note 24**).
7. Perform each reaction for three times. Analyze the results using GraphPad software. The results further prove that Glu564 plays a crucial role in the hydrolysis and none of the three modules is dispensable for the activity of $LytB_{CAT}$.

4 Notes

1. All pdb files mentioned here are download from the RCSB protein data bank (http://www.rcsb.org/pdb/), unless otherwise specified.
2. The used version of CCP4 software needs to be compatible with the computer system. Otherwise, the running may fail.
3. The RBB powder should be dissolved in 0.25 M NaOH, but not in water, for NaOH supplies an alkaline buffer system for the labeling of PG.
4. The primary sequence analysis of LytB or the structural information of GH73 family can also be obtained in the Pfam Homepage (http://pfam.xfam.org/) by sequence search or key word search, respectively.
5. The pdb file used for superposition should contain only the residues of protein itself, but not other molecules, such as water molecules, glycerol molecules and so on.
6. The job title can be blank, and there is no need to change other default options. Better not to check the "combine superposed coordinates with fixed coordinates in output PDB file" option. If you check this option, the two superimposed structures will be combined in the output pdb file, which is not convenient for graphing.

7. This step can be performed simultaneously with **step 3** in Subheading 3.1.
8. Run the server once for only one structure. When the search has finished, you will receive an email notification. It is better to give each running a job title when doing more than one structural comparisons successively.
9. Many superimposed structures with different Z scores will be given, in which many are redundant. A higher Z score means a structure more similar to the input structure. Summarize the hits and consider the functional relationship with the input structure.
10. The output of the second all-β module with the Dali sever includes several functionally unrelated proteins with a Z-score of ≤2.5. It is hard to classify the second all-β module to any family of structure-known proteins.
11. The ASTRAL database has now been integrated into the new SCOPe website (http://scop.berkeley.edu/). Go to the new website to get the new versions of both SCOPe and ASTRAL.
12. There could be many structures in every class, so it is necessary to analyze them carefully and patiently. With regard to the "all beta proteins" class, consider the number of strands in each fold first.
13. Receive the valid token immediately or several minutes later. A valid token could be used for five PRODRG runs.
14. If the Java version of the browser is outdated, the JME window may display with error. The JME help is in the bottom of the website to help draw the molecule. Pay attention to the chirality of the molecule.
15. Download the PDB file in four formats: all H's, polar/aromatic H's, polar H's only and no H's, which differ from each other in the number of H atoms in the coordinate file. It is better to choose the all H's format.
16. The size of the grid box must cover the entire active site and allow the ligand to move freely.
17. Delete the atomic coordinates of the receptor before inputting the atomic coordinates of the ligand. Or reopen the AutoDock Tools software and then input the ligand.pdb. Choosing torsion depends on your request.
18. The exhaustiveness parameter sets the number of runs, telling the program how hard to search. It is an optional setting with a default value of 8.
19. When invoking, the $LytB_{CAT}$.pdbqt, the TSPP.pdbqt, and the built txt file must be saved in the same folder.

20. The AutoDock Vina may give a set of docked poses. The pose with the highest affinity may not be the most rational one. Compare different metrics, such as the interaction between receptor and ligand, free energy of binding, RMSD, van der Waals, and so on, in a general consideration when choosing the final simulated model.
21. Discard the supernatant carefully without touching the precipitate.
22. The concentration of protein and the RBB-labeled Δ*lytB* PG reminded here means the final concentration in the reaction mix. The volume of the reaction mix can be enlarged to 200 μL. Keep the protein and the RBB-labeled Δ*lytB* PG on ice before starting the reaction. It is better to add the RBB-labeled Δ*lytB* PG to the reaction system in prior of adding protein.
23. Avoid disturbing the precipitate when pipetting the supernatant.
24. Use the same volume reaction buffer without protein as the blank control.

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