

Virtual Screening of Inhibitors for Chitosanases EAG1

Xiangyang Xu*, Zaiwei Song, Yunchao Yin, Faguo Zhong, Junying Song, Jiachao Huang, Wangli Ye, Peng Wang

Zaozhuang Jienuo Enzyme Co., Ltd., Zaozhuang City, China

Email: *sdjienuo@163.com

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Abstract

Chitosanases EAG1 is a classical glycoside hydrolase from *Bacillus ehimensis*. The previous researches showed that this Chitosanases can not only hydrolyze the b1,4-glycosidic bonds of chitosan to COS in different sizes but also keep a high catalytic activity in organic, which was useful for producing chitooligosaccharides and GlcN for use in the food and pharmacological industries. While it is instable in the liquid state. This shortcoming seriously restricts its industrial application. Here we used the modeled structure of EAG1 and the molecular modeling software package to screen the free chemical database ZINC. Moreover, the strategies including “initial filter” and consensus scoring were applied to accelerate the process and improve the success rate of virtual screening. Finally, five compounds were screened and they were purchased or synthesized to test their binding affinity against EAG1. The test results showed that one of them could inhibit the enzyme with an apparent K_i of 1.5 μM . The result may take the foundation for further inhibitor screening and design against EAG1 and the screened compound may also help to improve the liquid stability of EAG1 and expand its industrial application.

Keywords

Chitosanases, Inhibitor, Virtual Screening

1. Introduction

Chitosanases belong to glycoside hydrolase families 5, 7, 8, 46, 75 and 80 and hydrolyse glucosamine polymers are produced by partial or full deacetylation of chitin [1]. They are of important industrial application in the utilization of the enormous chitosan and chitin substrates, available from sea-food-processing units, for the generation of the size-specific chitosan oligomers required particu-

larly in pharmaceutical industries [2]. In industrial enzyme applications, stability is a key factor. In order to improve the stability of enzymes, a number of protein engineering techniques, such as random mutagenesis, DNA shuffling, truncation and circularization, have been developed for improving the stability of proteins [2]. However, there was not any work focusing on improving liquid stability for Chitosanases by adding inhibitors. This technology had been used on protease. The prior art has dealt extensively with improving the storage stability, for example, by adding a protease inhibitor [3] [4] [5]. The application of high concentration liquid enzyme preparation in industry will become the development direction in the future.

VS is a technique to identify novel hits (*i.e.*, bioactive molecules) from large chemical libraries through computational means by applying knowledge about the protein target (structure-based VS) or known bioactive ligands (ligand-based VS). The ligand-based approaches utilize structure-activity data from a set of known actives in order to identify drug candidates for experimental evaluation. In the previous study, researchers have used virtual screening method to screen inhibitors of protease MP, and the obtained inhibitors have significantly improved the stability of the enzyme preparation [6]. EAG1 is a classical glycoside hydrolase from *Bacillus ehimensis*, it exhibits maximum activity at 50°C. Not only can it hydrolyze the b1,4-glycosidic bonds of chitosan to COS in different sizes but also keeps a high catalytic activity in organic solvents with metal ions [7]. In the previous researches, its tertiary has been modelled [8] and a disulfide bridge in flexible regions can improve EAG1's thermo-stability and catalytic efficiency [7]. However, it is unstable in liquid state, which is the common disadvantage as all enzymes.

In the present study, we focused on database screening, biological evaluation and mechanism illumination of inhibitors for Chitosanases EAG1. Our virtual screening approach was based on initial high through-put docking calculations performed on a library of about 23,000,000 commercially available compounds in the database of ZINC [9] [10]. The recompile software of Autodock4.2 was used for docking produce, consensus score, as well as the special filter strategy built based on the experimental and docking results of known inhibitors for Chitosanases were applied to improve the success rate of inhibitors finding. Three compounds were synthesized and assayed *in vitro*. One of them could inhibit EAG1 with apparent K_i with 1.5 mM, which could be used for further inhibition mechanism detection. This finding provided the bases for further selection and synthesis of more effective inhibitors for Chitosanases EAG1.

2. Material and Methods

Docking studies and the “initial filter” building

The structure of EAG1 [7] was modeled in the previous work and it was clarified that all of the residues were in the acceptable zone. The modeled structure was defined as the acceptor. The active site was defined using AutoGrid and the

center (37.286, 4.2688, 25.31) of the box was chosen according to the position of key residue in pocket. The grid size was set to $60 \times 60 \times 60$ points with grid spacing of 10 Å (Figure 1). The grid box included the entire binding site of the enzyme and the residues in active site were set flexible in the whole docking process. The small molecules were defined as ligands and the small molecular was set as flexibility ligand. That is to say, all rotational bonds were set free.

The Autodock4.2 program has been proved to be a robust approach with good docking accuracy and reliability in the docking of inhibitors [11] [12]. And the Autodock Tools (ADT) [13] was used for preparing molecules and all of the hydrogen was added by using REDUCE [14]. All of the docking decoys were clustered with cutoff 2 according to root mean square deviation (RMSD).

Previous studies of inhibitors for Chitosanases showed that some compounds had good capacities as Chitosanases inhibitors (Table 1). Hence, we used these compounds to train the characters and built an “initial filter” for the virtual screening. All of the character setting was just as the steps mentioned above. The tertiary structures of these compounds were listed in Figure 2. These structures were downloaded from Chemspider [15] (<http://www.chemspider.com/>).

Table 1. The experimental K_i and docking K_p , E_i values for compounds in training set.

Compounds	K_i (μ M)	Docking K_p (μ M)	Docking E_i (kcal/mol)
Benzalkonium chloride	2.36	8.89	-4.16
2-Hydroxy-5-nitrobenzyl bromide	0.42	2.85	-8.14
N-bromosuccinimide	0.07	0.93	-15.41
Sodium acetate	0.48	2.29	-8.62
p-chloromercuribenzoate	0.20	1.61	-9.54
p-hydroxymercuribenzoic acid	0.16	1.46	-10.69
2-Mercaptoethanol	0.02	0.49	-12.64
Diethyl pyrocarbonate	0.03	0.67	-13.05
Ethyl acetimidate	1.40	8.07	-5.03
EDTA	1.92	8.67	-5.69
Glucosamine	0.32	2.11	-8.14
Triton X-100	0.09	1.05	-14.48
Cysteine	1.82	8.51	-5.54
Tween 20	0.07	0.97	-14.01
guanidinium hydrochloride	0.38	2.74	-7.64
Tween 80	0.06	0.89	-13.80
Monoiodoacetate	0.91	5.72	-6.08

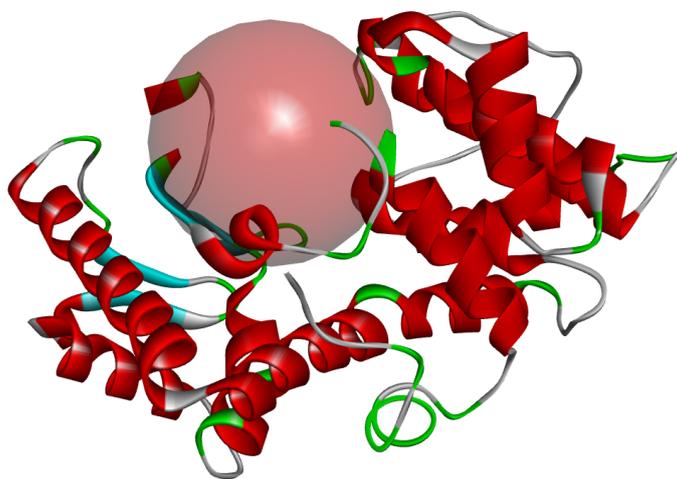


Figure 1. The docking zone. Structure of EDG1 was shown in cartoon and the docking zone was shown in red ball. The figure was generated by Discovery Studio Client 4.2.

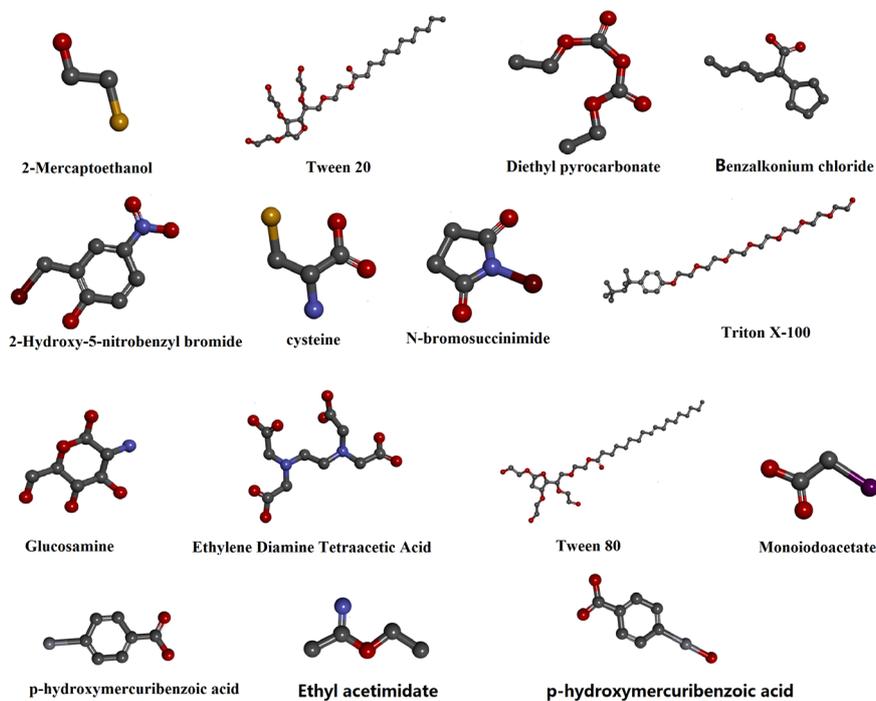


Figure 2. Chemical structures of inhibitors for Chitosanases in training set.

P-hydroxymercuribenzoic acid, 2-Mercaptoethanol, Diethyl pyrocarbonate, N-bromosuccinimide, Glucosamine, Triton X-100, 2-Hydroxy-5-nitrobenzyl bromide, Tween 20, Tween 80 and guanidinium hydrochloride, they inhibiting EAG1 with the K_i values from 0.02 to 0.48, were not effective inhibitors. Because the complex they formed were not stable. On the contrast, Benzalkonium chloride had strong inhibition ability and it was not suitable inhibitors to improve EAG1 stability. We docked these compounds to MP by using the Autodock4.2. The docking values of E_i and K_i of the favorite cluster were extracted and listed in **Table 1**. Considering the above factors, the restrictive condition of docking E_i

is not less than -6.00 kcal/mol and not greater than -5.00 kcal/mol, the docking K_i not less than $8 \mu\text{M}$ and not greater than $9 \mu\text{M}$ was set as the “initial filter” for EAG1 inhibitor selection.

Virtual docking screening combined with consensus scoring

The free commercially available compounds database ZINC, of about 2,300,000 compounds, was used as the docking library. The tertiary structures of these compounds included in the docking library were downloaded strictly from the database ZINC. Those compounds with both dock energy-score and K_i score meeting the selective conditions ($-6.000 \leq E_i \leq -5.000$, $8 \leq K_i \leq 9$) were chosen as the primary selection list. After this selection, about 5000 compounds were selected by docking scores from the pool of docking library.

The X-score [16] program, which computed the binding affinities of the given ligand molecules to their target protein, was applied to reevaluate the primary selected molecules following “initial filter” screening. The calculated score value could somewhat remove the score bias caused by single docking and was considered more accurate and possible to reflect the actual binding ability. The multiple conformations of 5000 compounds were then calculated and reevaluated by Xcscscore to generate the final “hit lists”. All compounds with calculated score higher than 5.0 and ranked in the top 500 for docking E_i and Xcscscore results were kept in the hit lists. Finally, the top three compounds were selected (Table 2) and purchased for further assay.

Chitosanases assay

Enzyme purification

EAG1 was purified by ultrafiltration membrane and Ni^{2+} -charged 1 ml His Trap FF crude column (GE Healthcare), According to the methods from Sheng *et al.* [7] Chitosanase activity was determined at 40°C by estimating the amount of the reducing ends of sugars using a modified dinitrosalicylic acid (DNS) method with glucosamine $\cdot\text{HCl}$ as the calibration standard. It was measured in 0.2 mL of reaction mixture that contained 100 mM acetate buffer, 0.2% chitosan, and suitably diluted enzyme. One unit of chitosanase was defined as the amount of enzyme required to liberate $1 \mu\text{mol}$ reducing sugar per min under the conditions described above. Three replicates were performed per analysis.

The effect of Inhibitors on EAG1 activity was studied at 40°C using chitosan as substrate. The enzyme was pre-incubated with the inhibitors for 5 min. The measurements were performed in 200 μL final volume, at 40°C in 100 mM acetate

Table 2. Docking scores, X-scores and experimental K_i values for candidates.

Compounds	Docking energy (kcal/mol)	Docking K_i	Xscore	K_i (μM)
ZINC00344328	168.04	-5.15	6.87	
ZINC20476815	124.22	-5.33	6.36	1.50
ZINC02961282	101.33	-5.45	6.21	

buffer (pH 6.0) at 50 ng/mL enzyme concentration. The Michaelis constant (K_m) for PPO was determined by Lineweaver-Burk [17] plots and K_i value was obtained from Dixon plot [8].

Inhibitors

The effect of Inhibitors on MP activity was studied at 30°C using Succ. Ala-Ala-Pro-Arg-AMC as substrate. The enzyme was pre-incubated with the inhibitors for 5 min. The measurements were performed in 200 μ L final volume, at 30°C in an enzyme assay buffer (50 mM Tris-HCl (pH 8.0), 10 mM CaCl_2 , 0.1 M NaCl) at 5 - 30 ng/mL enzyme concentration. The activities on fluorometric substrates were measured at 380 nm excitation and 460 nm emission wave lengths [18]. The Michaelis constant (K_m) for MP was determined by Lineweaver-Burk plots and K_i value was obtained from Dixon plot. Boric acid and its derivations induced competitive inhibition. To describe the competitive inhibition mechanism, the Lineweaver-Burk equation in double reciprocal form can be written as:

$$\frac{1}{v_i} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (1)$$

Secondary plots can be constructed from

$$K_m^{app} = \frac{K_m [I]}{K_i} + K_m \quad (2)$$

Then, the K_i , K_m , and V_{\max} values can be derived from the above equations. The secondary replots of the apparent K_m vs. [I] is linearly fitted, assuming a single inhibition site or a single class of inhibition site [19].

3. Results and Discussion

The compounds isolated from the virtual screening were tested for their inhibition effects on EAG1. The top three Xscore compounds (Table 2) from the hit list were selected and ordered (or synthesized). And then, their binding affinities were measured by the assay method as mentioned above. In our experiments, the inhibition affinities for the three compounds were tested. Among them, one selected compounds turned out to inhibit the activity of EAG1 efficiently. The K_i values for the compound were experimentally determined to 1.50 μ M. It was reversible inhibitors of EAG1.

Under the conditions employed in the present study, the hydrolysis follows Michaelis-Menten kinetics. The kinetic parameters for the enzyme have been obtained from Lineweaver-Burk plot (Figure 3, curve- \blacklozenge), and the results show that the K_m and V_m are respectively 4.97 μ M and 3.5772 μ M/min.

The kinetics of the enzyme in the presence of inhibitor was studied using double-reciprocal Lineweaver-Burk plots. The results (Figure 3) revealed that the value of V_m remained the same and the value of K_m increased with increasing inhibitor concentrations, indicating that the selected compounds induced competitive inhibition. Using Equations (1) and (2), the K_i was calculated.

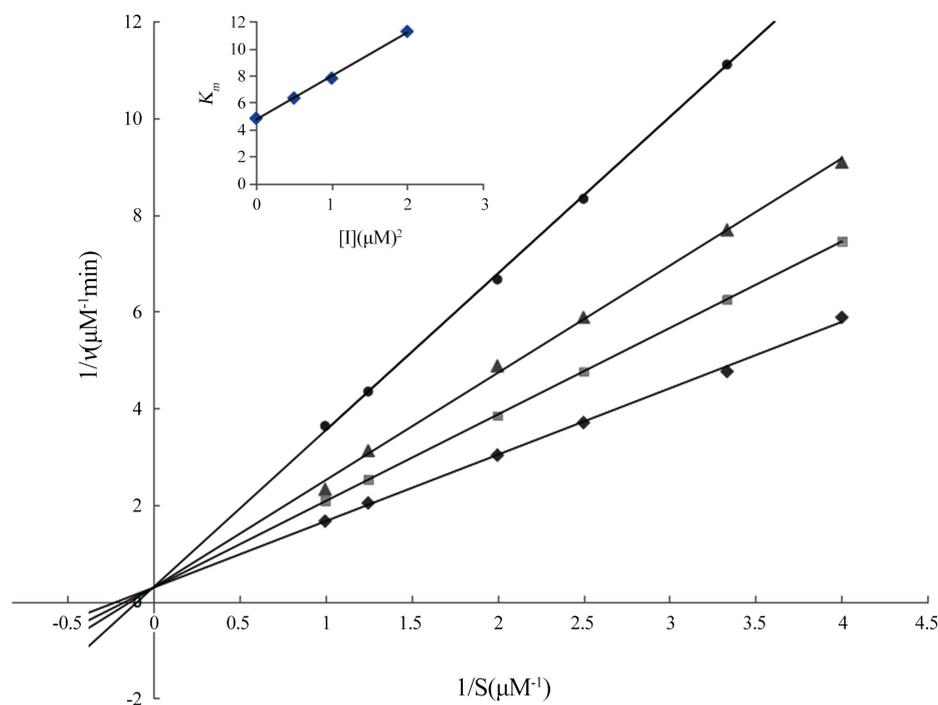


Figure 3. Plot of $1/v$ vs. $1/[S]$. The concentrations of ZINC20476815 were 0 ($-\diamond-$), 0.50 ($-\square-$), 1.00 ($-\triangle-$) and 2.00 ($-\bullet-$) μM , respectively. The label in figure is absolute error. The inset is the plot of K_m vs. $[I]$ to ascertain K_i .

4. Conclusion

Three potential compounds were selected and purchased for biological testing following the procedure of virtual screening and post-dock scoring. One of them was found to inhibit EAG1 in the millimole range. These active compounds served as the basis for developing inhibitors for EAG1. Overall, our results give another evidence that the effectiveness of the automated AutoDock program with the improved scoring function as a docking program for the *de novo* discovery of inhibitors. The method for finding EAG1's inhibitor in this study may further guide the development of inhibitors for EAG1.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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