RESEARCH ARTICLE

Analysis of Molecular Modeling and Molecular Docking of Beta-glucanase from Metagenomic Expression Library as *Candida* Antibiofilm Candidate

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ABSTRACT

Overgrowth of *Candida* tends to produce high levels of secondary metabolites affecting the immersion of infectious and degenerative diseases. Biofilm's existence as a virulence factor of *Candida* makes it challenging to overcome causing multidrug-resistant issues. Studies on the effectiveness of *Candida* antibiofilm drug candidates should be supported by data related to model structure and molecular interaction within the eradication process of biofilm through homology modeling and *in-silico* docking. This study aims to determine molecular interactions between 1,3-β-glucanase *Achatina fulica* in which the substrate is, through homology modeling and docking studies within the biofilm matrix eradication process. The alignment results show that mkafGlu1 is a new representative of the Glycoside Hydrolase 16 family (GH16) with EC 3.2.1.39. MKAFGlu1 enzymes are 1,3-1,6-β-glucanase able to cleave 1,3-β- dan 1,6-β-glycosidic bonds. This *in-silico* docking study also shows that mkafGlu1 has high specificity towards laminarin (substrate 1,3-1,6-β-glucanase) and that the enzyme of MKAFGlu1 works based on a retention mechanism in its reaction to the substrate. The result of the recombinant enzyme of 1,3-β-glucanase novel genes can hydrolyze the substrate of 1,3-β-glucan as one of the biofilm matrix components, opening up an opportunity for new medicine as an antibiofilm candidate.

Keywords: β-glucanase, Antibiofilm, Homology modeling, *In-silico*, Metagenomic.

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INTRODUCTION

Candidiasis is an infection condition caused by uncontrolled candida overgrowth.[1] *Candida albicans* is the most frequently infection-causing species compared to the other species.[2] *Candida albicans* pathogen fungi can cause a variety of diseases such as thrush, skin lesions, vulvovaginitis candidiasis, and gastrointestinal candidiasis.[3] The condition of Candida overgrowth tends to produce high metabolites, possibly harming the host body as it usually leads to the emersion of such degenerative diseases as Alzheimer's, Parkinson's, T2D, amyloidosis, cystic fibrosis, and crescent anemia.[4]

Treatment of candidiasis has been given through antifungal. However, even the most recent treatment using antifungals has only been able to reduce the population of *Candida*, not overcome it. The existing biofilm causes the antifungal to not be able to penetrate to work well. Biofilm matrix consisting of β-1,3-glucan, β-1,6-glucan, chitin, mannan, protein, and mannoprotein act out as a *Candida* protector from the host immune system which is later colonizing. This is the cause of *Candida* resistance towards almost all types of antifungal or multidrug resistance.[4,5]

Research results show that a consortium of glycoside hydrolase (GH) isolated from the digestive gland *Achatina fulica* is effective in hydrolyzing fungi biofilm matrix, particularly *C. Albicans*. This finding has opened up an opportunity to use GH from *A. fulica* and antifungal compounds to develop as a novel antifungal drug candidate to eradicate *Candida* biofilm on all types of candidiasis pathology.[4] In line with this, a previous study has proven that the results of expression of the novel gene encoding recombinant 1,3-β-glucanase can hydrolyze the substrate of 1,3-β-glucan as one of the components of biofilm matrix.[6]

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The biological process on the molecular level is a key to the development of GH *A. fulica* as a candidate for the antibiofilm drug of candidiasis.[7] Understanding the molecular level needs to be supported by the GH structure model and as well as its interaction in eradicating biofilm matrix. However, there has not been information related to molecular models and interactions to study the effectiveness of drug candidates. By utilizing the results of a previous study about the novel gene encoding 1,3-β-glucanase from the digestive gland metagenomic library *A. fulica*, this study conducts *in-silico* molecular development and functional analysis of β-glucanase from the metagenomic expression library as the *Candida* antibiofilm candidate.

METHOD

This study is conducted through modification of a method proposed by Kurniawati *et al.*[8] as presented in Figure 1. The construction phase of cDNA literature generally consists of cDNA ligation with vector TriplEx2, page packaging, library titer calculation, and determining the percentage of recombinant clones in the unamplified library.

Screening of Recombinant *Escherichia coli,* Based on 1,3-β-glucanase Activity

Screening of phage recombinant is conducted to the activity of 1,3-β-glucanase in which the substrate laminarin uses the congo red coloring. Positive plaque recombinant shows a color halo around the plaque. Phage recombinant with positive halo is converted to plasmid (pTriplEx2) through the SMART cDNA Library Construction Kid User Manual method.

Sequencing and Recombinant Clone Expression in *E. coli*

The conversion result of pTriplEx2 in *Escherichia coli* BM 25.8 is grown in media to be incubated within 12 to 16 hours at the temperature of 31°C. Isolation of recombinant plasmid follows the Thermo Scientific GeneJET Plasmid Midiprep Kit method modified with that proposed by Kotchoni *et al.*[9] but some of them are time consuming especially when extracting a large number of samples. Here, we developed a rapid protocol for plasmid DNA extraction based on the alkaline lysis method of plasmid preparation (extraction at pH 8.0 Analysis of DNA inserts with HinIII restricted enzymes based on the manual protocol of Assembly of Promega Restriction Enzyme Digestions. Sequencing of the recombinant plasmid is arranged automatically (Macrogen Inc., South Korea). Analysis of bioinformatics consists of sequencing analysis of deductive amino acids using Expasy Protparam of tool web server,[10] determining ORF through ORF finder NCBI web server, sequential alignment using Clustal Omega EMBL-EBI, and phylogenetic analysis using MEGA software.[11] Expression of β-glucanase genes in *E. coli* is conducted through positive cloning in *E. coli* BM 25.8 cultured in the 15 ml LB broth by 225 rpm shaker at the temperature of 31°C within 12–16 hours induced by IPTG. Cells are lysed by adding 200 µL of lysis buffer solution.

Table 1: Composition of amino acids in MKAFGlu1

No	*Amino acid*	*Number*	*Percentage (%)*
1.	Ala (A)	7	2,8
2.	Arg (R)	12	4,8
3.	Asn (N)	6	2,4
4.	Asp (D)	6	2,4
5.	Cys (C)	15	6,0
6.	Gln (G)	8	3,2
7.	Glu (E)	3	2,0
8.	Gly (G)	13	6,0
9.	His (H)	9	3,6
10.	Ile (I)	9	4,0
11.	Leu (L)	35	14,4
12.	Lys (K)	9	4,4
13.	Met (M)	5	2,0
14.	Phe (F)	17	6,8
15.	Pro (P)	15	6,4
16.	Ser (S)	38	15,6
17.	Thr (T)	8	3,6
18.	Trp (W)	4	1,6
19.	Tyr (Y)	6	2,4
20.	Val (V)	14	5,6

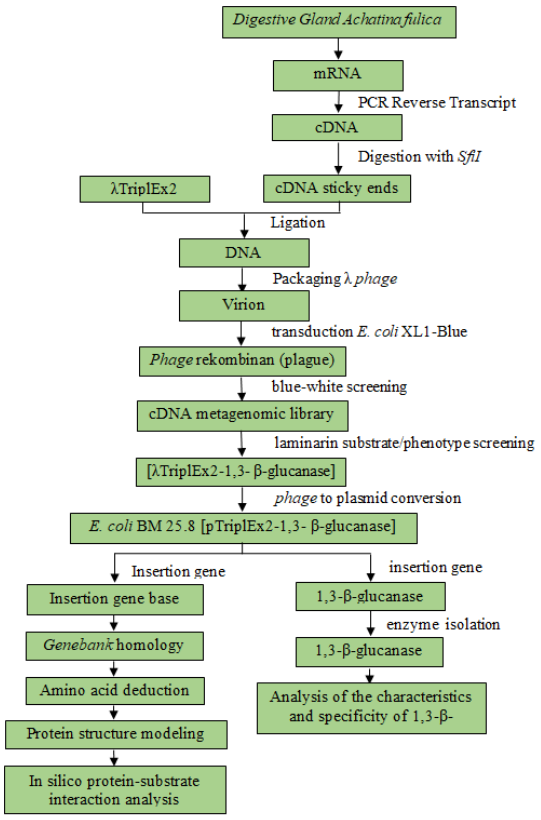

Figure 1: Strategies of in-silico functional and molecular analyses of beta-glucanase of the metagenomic library as a *Candida* antibiotic candidate

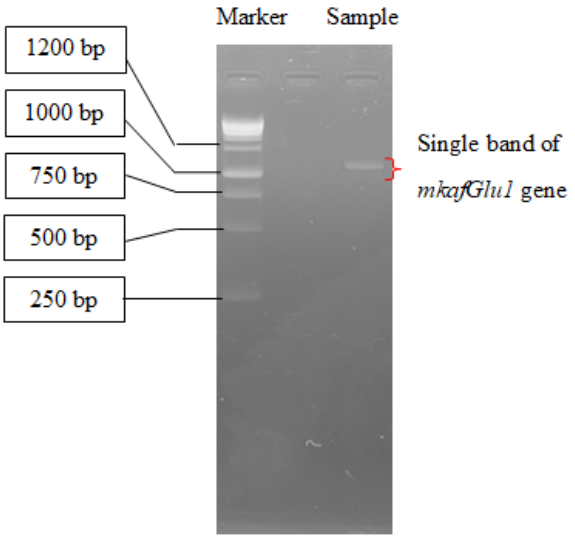

Figure 2: Verification of mkafGlu1 genes using PCR resulting in a single band of mkafGlu1 gene

Table 2: The affinity value for docking MKAFGlu1 with several glucan substrates.

No	Substrate compound	Affinity (kcal/mol)	Bond Type	EC Number
1.	Laminarin	- 7,6	β- 1→3; -1→6	EC 3.2.1.39
2.	Barley-β-glucan	- 5,1	β- 1→3; -1→4	EC 3.2.1.73
3.	CMC	- 3,0	β- 1→4	EC 3.2.1.4

Determining Specificity of Protein *In-silico*

Ligan structural design is acquired from the website of protein data bank (PDB) in .pdb files. In the meantime, molecular modeling of the protein 3D structural model is predicted to use the homology modeling program of I-TESSERI-TAssER first generates three-dimensional (3D).[12] Molecular docking is conducted through PyRx/AutoDock Vina[13] and its analysis is in the output of PyRx/AutoDock Vina in bond affinity (kcal/mol) between macromolecule-ligan which can be seen using PyMol and discovery studio software.

RESULTS AND DISCUSSION

Sequencing and Analysis of mkaf Glu1 Gene

A genetic clone that is successfully sequenced is later called mkafGlu1. This sequence has been listed on GeneBank with access code No. MH206587. The sequence of amino acids (AA) is deduced by MKAFGlu1 as a translation result of 717 nucleotides. The protein sequence consists of 239 AA residues including 60 AA N-terminal signal peptides (based on the prediction of the SignalP 3.0 online program).[14] In the meantime, the C-terminal lies in the AA 192-217 residues. The AA composition of MKAFGlu1 is predicted to use ExPASy online tool server10 as presented in Table 1.

Verification of the mkafGlu1 sequence is conducted through PCR through plasmid recombinant [pTriplEx2-mkafGlu1] and electrophoresis from the PCR results. Electrophoresis to the PCR results of mkafGlu1 insert genes is obtained through a single tape as big as 0.7 kb. The results of the verification strengthen the ORF mkafGlu1 data with nucleotide composition of as much as 717 bp (the electrophoresis is presented in Figure 2).

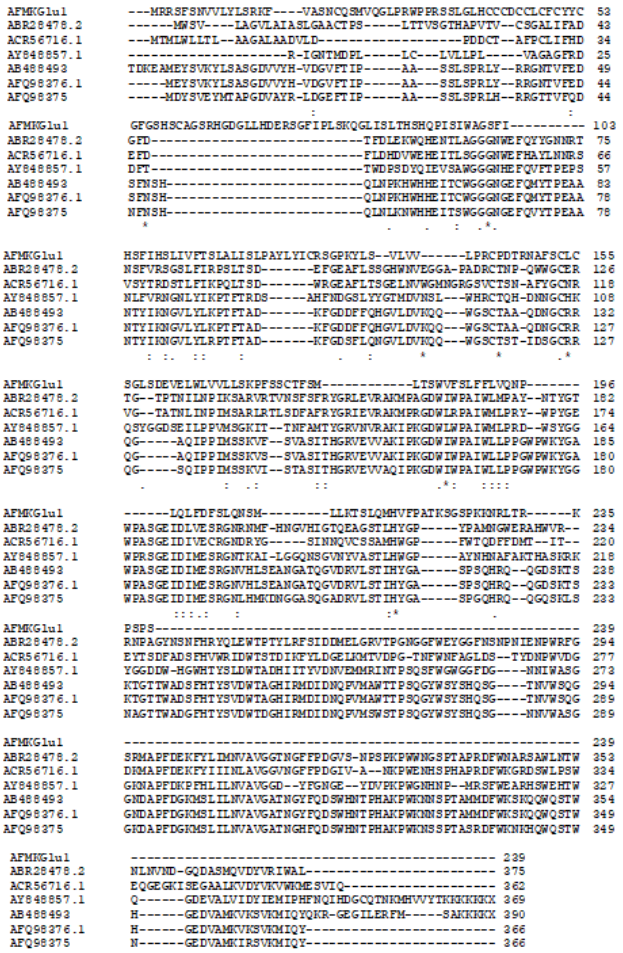

Figure 3: The 1,3-β-glucanase deduced amino acid sequence MKAFGlu1 is aligned with the 1,3-β-glucanase family 16 sequences of other species. The total conservation area is marked (*) and the partial conservation area is marked (.) and (:).

The results of multiple alignments of the AA sequences of MKAFGlu1 with BLASTp showed that MKAFGlu1 had a similarity of 45% (total score 16.2; E value 2.7) and 46% (total score 16.9; E value 1.4) with 1.3-β-glucanase from *Haliotis discus* hannai (GenBank access code No. AB488493) and *Prochoreutis sachalinensis* (Genbank access code No. AY308829), respectively. *H. discus* hannai is a type of sea slug and belongs to the mollusk group, while *P. sachalinensis* is a class of insects or insects. Both 1,3-β-glucanase from H. discus hannai and *P. sachalinensis* are members of the glycoside hydrolase family 16 (GH16). The results of multiple alignments of the total score and similarity to all AA showed that MKAFGlu1 had a low level of similarity (< 30%). The alignments of MKAFGlu1 to the AA sequences of several

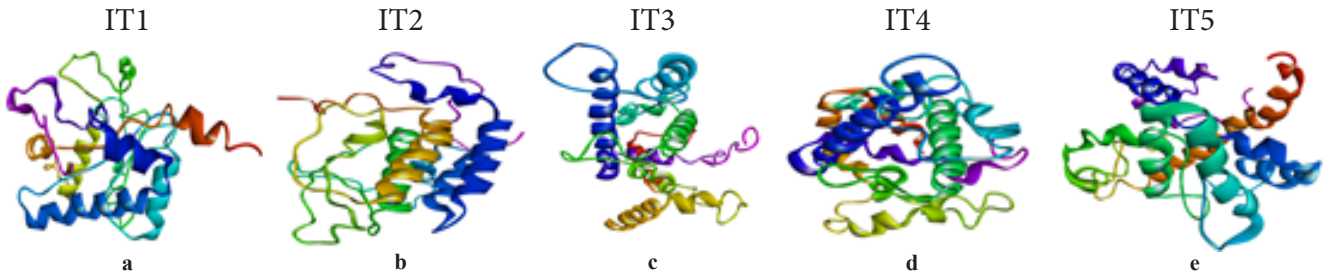

Figure 4: The 3-dimensional structure of MKAFGlu1 using I-TESSER. a. Structure of MKAFGlu1 model 1 with initials IT1; b. Structure of MKAFGlu1 model 2 with initials IT2; c. Structure of MKAFGlu1 model 3 with initials IT3; d. Structure of MKAFGlu1 model 4 with initials IT4; e. Structure of MKAFGlu1 model 5 with initials IT5.

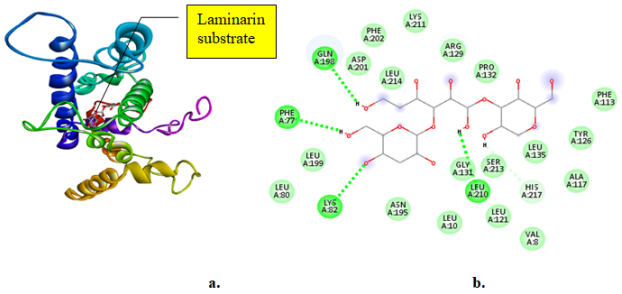

Figure 5: Docking of the MKAFGlu1-laminarin macromolecule. a. MKAFGlu1-laminarin docking with ribbon model visualization. b. Interaction of MKAFGlu1-laminarin with visualization using Discovery Studio software

GH16 members from other species are presented in Figure 3.

Amino acid residue analysis of the endo-1,3-β-glucanase GH16 showed that the two cysteine residues (Cys144 and Cys153) were conservative regions (Figure 3) for all endo-1,3-β-glucanases from mollusks including MKAFGlu1 as well as from other sources. This residue is possible to form disulfide bonds which are very important for the thermal stability of enzymes.[15]

The MKAFGlu1 sequence has a low homology when compared to the conservation region in the GH16 member sequences such as *Haliotis tuberculata* (GenBank access code No. AFQ98375.1), *Homoeocera gigantea* (GenBank access code No. AFQ98376.1), *Spodoptera frugiperda* (GenBank access code No. ABR28478.2), *Eriocheir sinensis* (GenBank access code No. ACR56716.1) and *Mizuhopecten yessoensis* (GenBank access code No. AY646657.1). Multiple alignments of amino acid sequences of endo-1,3-β-glucanase GH16 showed that the most conservative amino acid residue feature was "Glu-xxx-Asp-xxx-xxx-Glu" (in the red box Figure 3).[15,16] The first glutamate residue acts as a nucleophilic residue, while the second glutamate acts as an acid-base catalyst residue.[16] However, the most conserved amino acid residue was not found in MKAFGlu1, so it can be concluded that MKAFGlu1 is a new representation of endo-1,3-β-glucanase GH16.

Molecular Modeling MKAFGlu1 with Homology Modeling

Homology modeling with low sequence similarity (<30%), will increase the complexity of the modeling and be less reliable in predicting the resulting 3-dimensional structure.17,18

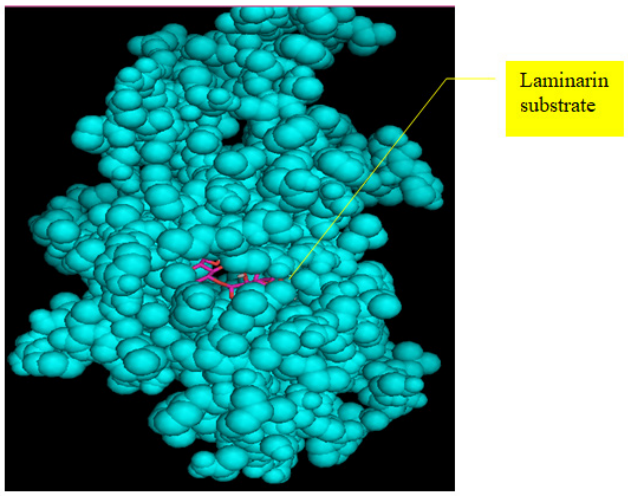

Figure 6: Visualization of molecular docking with Pymol software

The method used is homology modeling with 3ATG (GH16), 3AZX (GH16), and 3ILN (GH16) as templates using the I-TESSER web server. The I-TESSER is a web server with the best ranking in building protein structure predictions from experimental results or critical assessment of protein structure prediction (CASP)19 including CASP7, CASP8, CASP9, CASP10, and CASP11 experiments to build a prediction of quality protein structure.20 There are 5 models of the 3-dimensional structure of MKAFGlu1 which have been predicted with the help of I-TESSER. Each model is then given the initials IT1, IT2, IT3, IT4, and IT5.

The prediction of the secondary structure of MKAFGlu1 from I-TESSER is that the helix structure is located at positions AA between 1-6, 11-26, 40-52, 99-119, 149-155, and 178-217. The beta structure is in position AA between 85-89, 95-98, 125-128, 135-141, and 164-170. While the coil structure is in the AA positions between 27-39, 53-84, 90-94, 129-133, 142-148, 156-160, and 171-177. The secondary structure that forms the folding will build a tertiary structure. Prediction of the tertiary or 3-dimensional structure of MKAFGlu1 with I-TESSER is presented in Figure 4.

The 3-dimensional model structure built with I-TESSER has a C-score range from -5 to 2. The C-score is a score given to estimate the accuracy of the 3-dimensional model prediction based on the quality of the threading alignment process and

the smooth convergence of the simulated structure assembly. C-score -1.5 means that more than 90% of the prediction quality of the model is correct. The C-scores for IT1 to IT5 are IT1 -3.9, IT2 -4.59, IT3 -4.76, IT4 -4.87, and IT5 -5. Based on the C-score, IT1 is the prediction model with the best quality. In addition to the C-score, the quality of the model was also validated with the Ramachandran plot. Ramachandran plot parameters from IT1 to IT5 are IT1 47.5%, IT2 54.4%, IT3 67.3%, IT4 66.4%, and IT5 64.5%. Based on the Ramachandran plot21 parameters, the model with the best quality is IT3.

Molecular Docking MKAFGlu1

Docking analysis is a method used to identify electrostatic interactions between one molecule with another molecule (enzyme-substrate) in forming a stable bond. Broadly speaking, the docking method is divided into four parts, namely: 1. Preparation of MKAFGlu1 protein macromolecules, 2. Preparation of laminarin molecules as substrates, 3. Molecular docking between macromolecules and substrates, and 4. Analysis and visualization of molecular docking.

The MKAFGlu1 protein macromolecule used is the modeling result of the best model obtained from the I-Tesser modeling, namely IT3. The laminarin molecule was obtained from the PubChem Database of Chemical Molecules with the website address https://pubchem.ncbi.nlm.nih.gov/.[22] The laminarin used as the ligand was laminarin from Laminaria digitata with PubChem CID 71312131. The stability parameter between macromolecules and ligands was the affinity value. The more negative the affinity value, the more stable the complex formed. The best molecular docking result is the docking that produces the most negative affinity value (-7.6 kcal/mol). Docking results can also be used as a way to identify the most reliable model structure among the 4 prediction models based on the analysis of the Ramachandran plot value.

Docking is a simulation method that serves to determine the orientation between macromolecular receptors and ligands. This docking process uses a blind docking process, which is a docking process that is carried out without having to know the active side of the receptor. Blind docking can also be done without having to know the position of the MKAFGlu1 grid box. Molecular docking was performed using Pyrx protein-ligand docking software. Pyrx can predict ligand-binding receptors in a protein-ligand pocket. Pyrx visualization shows 9 protein-ligand poses and their binding affinities. The results of molecular docking in the first pose have the most negative affinity so that the first pose is the best docking pose between MKAGlu1-laminarin visualized with Discovery Studio in Figure 5.

The pocket residues surrounding the substrate include Val8, Leu10, Phe77, Leu80, Lys82, Phe113, Ala117, Leu121, Tyr126, Arg129, Gly131, Pro132, Leu135, Asn195, Gln198, Leu199, Asp201, Phe202, Leu2103, Lyu211, Ser21, and His217. Some bonds can be known, namely hydrogen bonds, covalent bonds, and non-covalent bonds (non-bonds). Covalent bonds and non-covalent bonds occur because of the interaction between the flexible ligand and the receptor. In general, electrostatic bonds and Van der Waals bonds occur. The bonds that occur can increase the affinity of the ligand. Hydrogen bonding resulting from the docking simulation between MKAFGlu1 macromolecules and laminarin ligands occurred at residues of Phe77, Lys82, Gln198, Leu210, and His217. In addition to hydrogen bonding, His217 also acts as a proton donor residue in the interaction between the MKAFGlu1 macromolecule and the laminarin ligand. While other residues contained in the catalytic pocket bind to the ligand via Van der Waals bonds.

Docking visualization (Figure 6) can be used to determine the mechanism of the hydrolysis reaction of glycosidic bonds. Hydrolysis of glycosidic bonds occurs through retention or inversion mechanisms. The similarity between the retention and inversion mechanisms is that they form an oxacarbenium ion at the reaction transition conditions. This reaction requires a carboxylic acid pair on the catalytic site. The difference between the retention and inversion mechanisms lies in the distance between the residues involved in the reaction process. In the inversion mechanism, the distance between the two carboxylic acid residues that act as donor/acid residues with nucleophilic/base residues is approximately 10Å and a double displacement reaction mechanism occurs. Meanwhile, in the retention mechanism, the distance between donor/acid residues and nucleophilic/base residues is approximately 5Å and a single displacement reaction mechanism occurs.[21]

The classic view presented by Kosland[23] is that inversion and retention reactions usually occur with the help of general acids and bases from the amino acid side chains of glutamic acid or aspartic acid. The diversity of catalytic reaction mechanisms that have been identified shows that Kosland's classical opinion is not always true.[24] Amino acids such as tyrosine, glutamic acid, aspartate, arginine, serine, threonine, glutamine, asparagine, and lysine have a major role in the accurate placement of substrates in macromolecular-ligand interactions and interact with ligands for specific activities.[24-26] The residues involved in the retention or inversion reactions are mostly in positions opposite to the glycosidic bonds.[27]

In MKAFGlu1, the acid and nucleophilic/base residues involved in the catalytic reaction of glycosidic bond breaking were predicted in the residues in the pocket of MKAFGlu1 residues (Figure 6). The docking results show that His217 residue acts as an acid residue that donates protons in the macromolecular-ligand interaction process. Identification of nucleophilic as well as residues on the active site, in general, catalytic residues are residues found in the conserved, polar, and hydrogen bonded region.[24] In MKAFGlu1, the estimated amino acids as catalytic residues are lysine 82 (Lys82) and glutamine 198 (Gln198). The average distance between the acid and nucleophilic/base residues involved in the catalytic reaction is 5.01Å so that the catalytic reaction of MKAFGlu1 glycosidic bond breaking follows a retention mechanism.

The stability parameter between macromolecules and ligands is the affinity value. The more negative the affinity

value, the more stable the complex formed. If the affinity value of MKAFGlu1 macromolecule docking with various substrates including laminarin, barley-β-glucan, Carboxymethyl Cellulose (CMC), and 1,4-β-glucan as a ligand, then laminarin has the most stable free energy value of other ligands. In Table 2 there are affinity values for docking results from several tested substrates.

The results of the docking analysis showed that MKAFGlu1 was specific for laminarin because the affinity value for laminarin was the most negative. MKAFGlu1 can hydrolyze laminarin which has 1,3;1,6-β-glucan monomer, but is less specific for barley-β-glucan which has 1,3;1,4-β-glucan monomer. MKAFGlu1 is a 1,3;1,6-β-glucanase protein capable of hydrolyzing 1,3-β-glucan and 1,6-β-glucan bonds, but has limited hydrolyzing action on 1,3-1,4-β-glucan in barley-β-glucan. Prediction of the lack of ability of MKAFGlu1 to hydrolyze 1,4-β-glucan is evident from the affinity value for CMC substrate with 1,4-β-glucan monomer only -3.0 kcal/mol. This proves that MKAFGlu1 is 1,3;1,6-β-glucanase with EC 3.2.1.39.

CONCLUSIONS

In this study, the gene encoding 1,3-β-glucanase was cloned using a library approach to cDNA metagenomic expression from the digestive system of Achatina fulica as a Candida antibiofilm candidate. Sequence alignment indicated that mkafGlu1 is a novel gene as a novel representation of glycoside hydrolase family 16 (GH16) with EC 3.2.1.39. MKAFGlu1 is a 1,3-1,6-β-glucanase that can cleave both 1,3-β- and 1,6-β- glycosidic bonds. *In-silico* results, deduced amino acid sequence mkafGlu1 had the highest specificity for laminarin (substrate 1,3-1,6-β-glucanase).

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