In silico phylogenetic, physicochemical, and structural characteristics of phytase enzyme from ten *Aspergillus* species

Karakteristik filogenetik, fisikokimia dan struktur in silico enzim fitase dari sepuluh spesies Aspergillus

Ridwan Putra FIRMANSYAH, Shobiroh NUUR'ALIMAH, I Made ARTIKA & Popi Asri KURNIATIN[*)]

Department of Biochemistry, FMIPA IPB University. Jl. Raya Dramaga, Bogor 16680, Indonesia

Abstrak

Asam fitat merupakan senyawa kimia yang terdiri dari inositol dan asam fosfat serta merupakan senyawa antinutrien yang terdapat pada bahan pakan unggas dari tanaman serealia. Fitase merupakan enzim yang dapat menghidrolisis ikatan fosfoester pada asam fitat dan menghasilkan fosfat anorganik serta ester fosfat. Aspergillus *merupakan salah satu genus kapang yang dapat menghasilkan fitase dan telah banyak digunakan dalam produksi fitase karena mudah dibudidayakan. Penelitian ini bertujuan untuk membandingkan struktur, sifat fisikokimia dan hubungan kekerabatan filogenetik fitase dari beberapa spesies* Aspergillus sp. *secara in silico sebagai langkah penyaringan awal untuk mendapatkan fitase yang paling cocok diaplikasikan pada pakan unggas. Pohon filogenetik diperoleh menggunakan MEGA 11 dan karakteristik fisikokimia menggunakan ProtParam. Struktur protein dimodelkan dengan AlphaFold. Struktur fitase kemudian di-docking dengan asam fitat menggunakan YASARA Structure. Hasil penelitian menunjukkan bahwa fitase 1QFX dari* Aspergillus niger*, P34755 dari* A. awamori *dan D5HQ11 dari* A. ficuum *mempunyai kemiripan yang tinggi baik dari segi filogenetik, sekuen, sifat fisikokimia dan struktur proteinnya. Hasil docking ketiga struktur fitase tersebut menunjukkan bahwa fitase 1QFX memiliki nilai ΔG paling negatif dan Kd terkecil yang menunjukkan afinitas tertinggi terhadap substrat asam fitat. Dari ketiga struktur fitase yang telah dibandingkan dan di-docking dengan asam fitat, fitase 1QFX dari* A. niger *merupakan fitase yang paling cocok untuk diaplikasikan pada pakan unggas.*

[Kata kunci: asam fitat, penambatan molekuler, pemodelan struktur, superpose*]*

Abstract

Phytic acid is a chemical compound consisting of inositol and phosphoric acid and is an antinutrient compound found in monogastric poultry feed ingredients made from cereal crops. Phytase hydrolyzes phosphoester bonds in phytic acid, releasing inorganic phosphate and phosphate esters. *Aspergillus* is a genus of molds that produce phytase and has been widely used in phytase production because they are easy to culture. This study aims to compare the structures, physicochemical characteristics, and phylogenetic relationships of phytases from several species of *Aspergillus* in silico as an initial screening step in obtaining the most suitable phytase to be used in poultry feed. Phylogenetic trees were constructed using MEGA 11 and physicochemical characteristics were analyzed using ProtParam. Protein structures were modeled with AlphaFold. The phytase structures were then docked with phytic acid using the YASARA Structure. The results showed that phytase 1QFX from *Aspergillus niger*, P34755 from *A. awamori,* and D5HQ11 from *A. ficuum* have very high similarity in terms of phylogenetics, sequences, physicochemical characteristics, and protein structures. The docking results from the three phytase structures showed that phytase 1QFX has the most negative ΔG value and the lowest Kd, which indicated the highest affinity to the phytic acid substrate. This research concludes that among the three phytase structures that have been compared and docked with phytic acid, phytase 1QFX from *A. niger* is the most suitable to be applied to poultry feed.

[Keywords: phytic acid, molecular docking, structure modeling, superpose]

[*)] Corresponding author: popi_asri@apps.ipb.ac.id

Grains from legume and cereal groups are widely used as raw materials in animal feed production. The use of grains in animal feed provides many advantages because they contain complete nutrients such as protein, carbohydrate, fat, crude fiber, Fe, K, and Ca that meet the daily nutritional needs of animals, stimulate growth, and thus improve farm productivity. In addition, grains are easy to obtain due to the large supply from agricultural businesses (Purnamasari et al., 2018). However, the use of grain as raw material in animal feed production has a drawback due to the phytic acid content in plant seeds (Silvia et al., 2016).

Grains contain a high level of phytic acid, a compound with antinutrient properties (Cominelli et al., 2020). It can reduce the digestibility of feed in livestock. Phytic acid (myo-inositol 1,2,3,4,5,6-hexakiphosphate) is a chemical compound with the formula $C_6H_{18}O_{24}P_6$ and is composed of six phosphate groups bound to an inositol structure. Phytic acid is the main storage form of phosphate in plants together with its precursors and derivatives. According to Rasyid et al. (2017), phytic acid is an anti-nutritional factor that is often found in grains which can disrupt or eliminate the use of feed ingredients, affect the physical and physiological condition of animals, and tend to have detrimental effects on animal productivity. Phytic acid has a nutrient removal mechanism because it is an inhibitor of various digestive enzymes and binds to various monosaccharides, complex peptides, and metallic minerals, thereby reducing the overall feed's nutritional availability. The high content of phytic acid in feed can cause phosphorus deficiency in livestock (Yanuartono et al., 2019).

One way to eliminate phytic acid content in animal feed is to use the phytase enzyme. Phytase or myo-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8.) is a hydrolase enzyme belonging to the phosphatase group. This enzyme catalyzes the breaking of the phosphoric ester bond between the phosphate group and inositol in phytic acid, releasing myo-inositol and inorganic phosphate (Figure 1). In addition, phytase can also produce products in the form of low myo-inositol phosphatase. Phytase is an extracellular enzyme (Nurhikmah, 2017). The phytate hydrolysis activity catalyzed by phytase causes the loss of the chelating properties and strong binding ability of phytate. This eliminates the antinutrient properties of phytate making the enzyme potential to be used in animal feed preparation to increase nutritional content. Phytase is grouped into 4 groups based on its phytate hydrolysis mechanism, namely histidine acid phytase, protein tyrosine phytase, purple acid phytase, and β-propeller phytase. Histidine Acid Phytase (HAPhy) is a phytase that has optimum hydrolytic activity in acidic conditions and is found in many animals, plants, and several microorganisms (Lei et al., 2013).

One group of microbes that produce phytase *Aspergillus* is suitable to be applied in poultry feed to increase its nutritional value. *Aspergillus* is widely distributed in both tropical and subtropical areas and has a very high species abundance in nature. In addition, various *Aspergillus* species have also been used by humans, especially in the conventional fermented food industry such as soy sauce, miso, and tauco (Daugelaite et al., 2013). According to Mizana et al. (2016), *Aspergillus* sp. has a high sporulation rate and is easy to culture in the laboratory, making it easier to use in phytase enzyme production on a laboratory scale. It is therefore necessary to conduct studies to compare the physicochemical characteristics among phytases from several *Aspergillus* species to screen the most suitable enzyme for the feed industry.

This research focuses on in silico characterization of *Aspergillus*. Previous research has proven the ability of *Aspergillus* isolates to produce phytase to hydrolyze phytic acid. However, limited studies have been carried out on the comparison of phytase structure and characteristics from various *Aspergillus* species. The present research aims to compare the
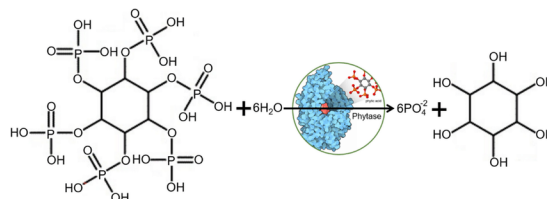

Figure 1. Hydrolysis of phytic acid by phytase (Coban et al., 2017)
Gambar 1. Hidrolisis asam fitat oleh fitase (Coban et al., 2017)

phylogenetic relationships, physicochemical and structural characteristics of phytases from several commonly cultured *Aspergillus* species in silico. The analyses include gene level and primary structure analysis, prediction and comparison of three-dimensional protein structures of phytases followed by molecular docking with phytic acid. This study is an initial screening step to select the most suitable phytase for application in the poultry feed industry.

Material and Methods

Tools and materials

The tools used in this research were a laptop with HP 14s-dk0073AU specifications with an AMD A4-9125 Dual-Core processor and 4 GB DDR4-1866 RAM, Windows 10 Home Single Language 64 operating system, Molecular Evolutionary Genetics Analysis software version 11.0 (MEGA11), Discovery Studio Visualizer 2017 R2 Client, LigPlot+, YASARA Structure, PyMOL, PROCHECK, ProtParam, Clustal Omega, and AlphaFold.

Fifteen amino acid sequences of phytase enzyme from ten *Aspergillus* species were downloaded from UniProtKB and RCSB Protein Data Bank (PDB) in FASTA format (1IHP, 1QFX, 1QWO, 1SKB, 3K4Q, A0A1L9VZ88, A0A1S9D908, A0A1S9DYU6, A0A3M7JKT4, A0A7G5K0G3, B3VPB2, D5HQ11, O00093, P34755 and Q0CLV1). In addition, fifteen gene sequences encoding each protein were also downloaded from EMBL-EBI bank website (https://www.ebi.ac.uk/).

Signal peptide removal

A total of five phytase sequences were downloaded from the RCSB PDB (https://www.rcsb.org/) and ten others were downloaded from UniProtKB (https://www.uniprot. org/). The fifteen gene sequences of these proteins were downloaded from the EMBL-EBI bank site. Next, FASTA file preparation is carried out by determining and removing the signal peptide at the beginning of each protein sequence. Signal peptide determination was carried out using SignalP 6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/) (Gómez et al., 2017).

Phylogenetic analysis

The initial alignment was carried out using protein BLAST (basic local alignment search tool) or BLASTp from NCBI (https://www.ncbi.nlm.nih.gov/) to determine the level of similarity between protein sequences. After obtaining the BLAST results, the alignment was followed by multiple sequence alignment (MSA) analysis to obtain data related to

similarities between the phytase sequences. MSA analysis was carried out using ClustalW platform on Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). All FASTA protein sequences were then used to build the phylogenetic tree using MEGA11 software (Mizana et al., 2016).

Prediction of physicochemical character

Physicochemical characteristics analysis of phytase was carried out on the Expert Protein Analysis System (ExPASy) Molecular Biology Server portal. The analysis was carried out using ProtParam tool (https://web.expasy.org/protparam/) (Pramanik et al., 2018).

Protein structure modeling and comparison

Protein structure modeling was carried out to model the phytase protein structure before molecular docking. The modeled structures are from the top three phytases with the highest similarity in terms of phylogenetics, sequence alignment, and physico-chemical parameters, namely 1QFX, D5HQ11, and P34755. The structure modeling was carried out using AlphaFold (https://alphafold.ebi. ac.uk/). The quality of the modeled structures was then analyzed using Ramachandran plots obtained from PROCHECK site (https://saves.mbi.ucla.edu/) (Irfanuddin, 2018).

A comparison of the structure models of 1QFX, D5HQ11, and P34755 was carried out using PyMOL software (https://pymol.org/). PyMOL was used to perform superimpose analysis. The root mean square deviation (RMSD) value of all atoms is used as a parameter to determine the quality of the structure models. In addition, the aligned positions of the structures were also being compared and visualized (Akdel et al., 2020).

Molecular docking of phytase

Molecular docking of phytase enzyme and phytic acid test ligand was carried out using YASARA Structure software. The protein file in *.sce format as a result of the preparation was opened and the docking was targeted at the active sites of the known phytase proteins, namely Arg62, His63, Arg66, Asp75, Arg156, Glu272, His318, and Asp319. Molecular docking was done by moving the doc_run.mcr file to the working folder and editing the file 25 times during the docking process. The output files (in *.yob and *.txt format) containing the binding free energies, dissociation constants, and amino acid residues data were used as the basis for further analysis. Visualization and analysis of amino acid residue interactions in the 2D molecular docking results were

carried out using LigPlot+ software. PyMOL software was used to analyze the interaction between ligand and protein and to visualize the position of the ligand within the protein binding site (Ramdani et al., 2019).

Results and Discussion

Alignment and phylogenetic analysis

The phytase protein sequences used in this research were downloaded from UniProt and PDB databases. A total of 15 phytase sequences were screened to determine the phytase with the best characteristics (Table 1). The 15 samples were chosen from ten species of *Aspergillus* widely cultured in laboratories. It is necessary to carry out a sequence alignment to compare similarities and residues in each selected enzyme sequence. Sequence alignment analysis is the comparison of two or more DNA or protein sequences to determine the level of similarity among the sequences. One way to analyze the similarities among protein or DNA sequences is by using BLAST, a bioinformatics tool closely linked to NCBI databases. BLAST is used to search for proteins or DNA entries which sequences are similar to our sequence of interest. BLAST protein (BlastP) is

specifically used to search for similar proteins in NCBI protein databases (Karunasekera, 2013).

The alignment of BlastP results using 1QFX phytase from *A. niger* as the query protein showed that it had an exact similarity to P34755 from *A. awamori* (Figure 1). The P34755 sequence also has the highest query cover and percent identity score. The D5HQ11 sequence from *A. ficuum* also has the same query cover and good percent identity to 1QFX and P34755. The lowest percent identity (24.22%) is shown by A0A1L9VZ88 from *A. glaucus*. The BlastP utilizes several other parameters such as percent identity and query cover to determine the level of protein homology. Percent identity represents the similarity of the query and the target sequences (how many characters in each sequence are identical). The higher the percent identity score, the more similar the query sequence is to the target sequence. Query cover denotes how much of the query sequence is overlapped by the target sequence. The query cover is 100% when the target sequence covers the entire query sequence. This provides the user with sequence length information relative to each other. Thus, a higher query cover indicates that the target sequence is more similar to the query sequence (Pratiwi et al., 2020).

Table 1. BlastP results showing 15 homologous *Aspergillus* phytases
Tabel 1. Hasil BlastP menunjukkan 15 fitase dari Aspergillus *yang homolog*

Protein accesion code *Kode aksesi protein*	Species *Spesies*	Query cover *Cakupan kueri*	Percent identity *Persentase kemiripan*
P34755	*A. awamori*	100%	100.00%
1QFX	*A. niger*	100%	100.00%
D5HQ11	*A. ficuum*	100%	97.39%
A0A1S9D908	*A. oryzae*	89%	35.47%
A0A7G5K0G3	*A. flavus*	86%	29.64%
1QWO	*A. fumigatus*	80%	27.10%
1SKB	*A. fumigatus*	80%	27.10%
3K4Q	*A. niger*	76%	26.99%
1IHP	*A. ficuum*	76%	26.99%
B3VPB2	*A. japonicus*	71%	26.78%
O00093	*A. nidulans*	83%	26.48%
A0A1S9DYU6	*A. oryzae*	76%	25.96%
A0A3M7JKT4	*A. flavus*	76%	25,83%
Q0CLV1	*A. terreus*	75%	25.52%
A0A1L9VZ88	*A. glaucus*	86%	24.22%

```
sp|Q0CLV1|PHYA_ASPTN              YAPYFSLQD-ESPFPLDVPDDCQITFVQALARHGARYPTDSKTKAYAATIAAIQK-NATT    89
sp|O00093|PHYB_EMENI              YSPYFSIEQ-ESAISEDVPHGCEVTFVQVLSRHGARYPTESKSKAYSGLIEAIQK-NATS    87
tr|B3VPB2|B3VPB2_ASPJA            YAPFFSLAN-ESAISPDVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQ-NATT    87
1IHP_1|Chain                     YAPFFSLAN-ESVISPEVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQ-NATT    79
3K4Q_1|Chains                    YAPFFSLAN-ESVISPEVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQ-NATT    85
tr|A0A1L9VZ88|A0A1L9VZ88_ASPGL   YSPYFAVGS-EPGLSGDVPSECQITFANVLSRHGARYPTASKSSKYSSLIEDIQN-NATS   165
1QWO_1|Chain                     YSPFFSLED-ELSVSSKLPKDCRITLVQVLSRHGARYPTSSKSKKYKKLVTAIQA-NATD    84
1SKB_1|Chain                     YSPFFSLED-ELSVSSKLPKDCRITLVQVLSRHGARYPTSSKSKKYKKLVTAIQA-NATD    81
tr|A0A1S9DYU6|A0A1S9DYU6_ASPOZ   YSPYFSVDD-ESSLSEDVPDHCQVTFAQVLSRHGARYPTKSKSEKYAKLIKAVQH-NATS    88
tr|A0A3M7JKT4|A0A3M7JKT4_ASPFL   YSPYFSVDD-ESSLSEDVPDHCQVTFAQVLSRHGARYPTKSKSEKYAKLIKAVQH-NATS   137
tr|A0A7G5K0G3|A0A7G5K0G3_ASPFL   NSPWFAGPN-VNKIASTVPEECSVDQAIYIVRHGSRYPDPGAYQEWEDLHNAFQS-AEYR    67
tr|A0A1S9D908|A0A1S9D908_ASPOZ   NGPWIEKIDELETPGLEPPKGCVIDQVHMISRHGERYPTKSAGSRHLALLNRIKE-ANVV   107
1QFX_1|Chains                    NGPYSERVSY--GIARDPPTSCEVDQVIMVKRHGERYPSPSAGKDIEEALAKVYSINTTE    90
sp|P34755|PHYB_ASPAW             NGPYSERVSY--GIARDPPTSCEVDQVIMVKRHGERYPSPSAGKDIEEALAKVYSINTTE    90
tr|D5HQ11|D5HQ11_ASPFI           NGPYSERVSY--GIARDPPTGCEVDQVIMVKRHGERYPSPSAGKSIEEALAKVYSINTTE    90
                                  .*:    .          *  * :  .  : *** * *  . .       .

sp|Q0CLV1|PHYA_ASPTN              RAADSSRVHESAEKFVEGFQNARQGDPRANPHQPSPRVDVVIPEGTAY-NNTLE-HSICT   197
sp|O00093|PHYB_EMENI              RASGSDRVVASAEKFINGFRKAQLHDHGSK--RATPVVNVIIPEIDGF-NNTLD-HSTCV   193
tr|B3VPB2|B3VPB2_ASPJA           RSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSS-NNTLD-PGTCT   195
1IHP_1|Chain                     RSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSS-NNTLD-PGTCT   187
3K4Q_1|Chains                    RSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSS-NNTLD-PGTCT   193
tr|A0A1L9VZ88|A0A1L9VZ88_ASPGL   RASGSDRVIASGEKFIEGFQSAKTNDTHAKTNQTTPSVNVVIAEGTEY-NNTLD-HSICT   273
1QWO_1|Chain                     RASGSDRVIASGEKFIEGFQQAKLADPGAT-NRAAPAISVIIPESETF-NNTLD-HGVCT   191
1SKB_1|Chain                     RASGSDRVIASGEKFIEGFQQAKLADPGAT-NRAAPAISVIIPESETF-NNTLD-HGVCT   188
tr|A0A1S9DYU6|A0A1S9DYU6_ASPOZ   RASGSDRVIASGEKFIEGFQKAKLGDSKSKRGQPAPIVNVVITETEGF-NNTLD-HSLCT   196
tr|A0A3M7JKT4|A0A3M7JKT4_ASPFL   RASGSDRVIASGEKFIEGFQKAKLGDSKSKRGQPAPIVNVVITETEGF-NNTLD-HSLCT   245
tr|A0A7G5K0G3|A0A7G5K0G3_ASPFL   WANDYQRTIDSARLFARGYLGPNASYGD-------VYV--VDADASGAAGNSLATSDQCP   174
tr|A0A1S9D908|A0A1S9D908_ASPOZ   WASECNRVIETAQHFASGFFGLDWEKTE-------KAALEVIPETLERGADTLTPGDTCP   220
1QFX_1|Chains                    FSSGYGRVIETARKFGEGFFGYNYSTN---------AALNIISESEVMGADSLTPTCDTD   200
sp|P34755|PHYB_ASPAW             FSSGYGRVIETARKFGEGFFGYNYSTN---------AALNIISESEVMGADSLTPTCDTD   200
tr|D5HQ11|D5HQ11_ASPFI           FSSGYGRVIETARKFGEGFFGYNYSTN---------AALNIISESEVMGADSLTPTCDTD   200
                                  :   *. :.. *  *:                  : :       ::*

sp|Q0CLV1|PHYA_ASPTN              HSPVHDHTCVNNTLDANPATFPLNATLYADFSHDSNLVSIFWALGLYNGTKALSQTTVED   369
sp|O00093|PHYB_EMENI             QSPVQDNTSTNHTLDSNPATFPLDRKLYADFSHDNSMISIFFAMGLYNGTQPLSMDSVES   365
tr|B3VPB2|B3VPB2_ASPJA           HSPVHDDTSSNHTLDSNPATFPLNSTLYADFSHDNGIISILFALGLYNGTKPLSTTTVEN   367
1IHP_1|Chain                     HSPVHDDTSSNHTLDSSPATFPLNSTLYADFSHDNGIISILFALGLYNGTKPLSTTTVEN   359
3K4Q_1|Chains                    HSPVHDDTSSNHTLDSNPATFPLNSTLYADFSHDNGIISILFALGLYNGTKPLSTTTVEN   365
tr|A0A1L9VZ88|A0A1L9VZ88_ASPGL   QTPVQDHTSTNRTLDSKPNTFPLNTTLYADFGHDNGMVSIFFALGLYNSTQDLSRDTIES   446
1QWO_1|Chain                     RSPVQDHTSTNLVSNPATFPLNATMYVDFSHDNSMVSIFFALGLYNGTEPLSRTSVES   363
1SKB_1|Chain                     RSPVQDHTSTNSTLVSNPATFPLNATMYVDFSHDNSMVSIFFALGLYNGTEPLSRTSVES   360
tr|A0A1S9DYU6|A0A1S9DYU6_ASPOZ   KSPVKDHTTTNNTTLDSNPATFPLNATLYADFSHDNTMTSVFFALGLYNTTEPLSQTSVQS   368
tr|A0A3M7JKT4|A0A3M7JKT4_ASPFL   KSPVKDHTTTNNTTLDSNPATFPLNATLYADFSHDNTMTSVFFALGLYNTTEPLSQTSVQS   417
tr|A0A7G5K0G3|A0A7G5K0G3_ASPFL   EGPSATAEKGNDTLQL--------PPLVVAFTHDNQLNELASLLGVFDDQKP---LASNK   331
tr|A0A1S9D908|A0A1S9D908_ASPOZ   SGP-EA------------------GTMFFSFVHDGDIAPLVTAMDILKDPKYDPFLPTTH   376
1QFX_1|Chains                    QGPKEA------------------GSLFFNFAHDTNITPILAALGVLIPNED---LPLDR   342
sp|P34755|PHYB_ASPAW             QGPKEA------------------GSLFFNFAHDTNITPILAALGVLIPNED---LPLDR   342
tr|D5HQ11|D5HQ11_ASPFI           QGPKEA------------------GSLFFNFAHDTNITPILAALGVLIPDED---LPLDR   342
                                  *                  :  * ** : :   :.:   .
```
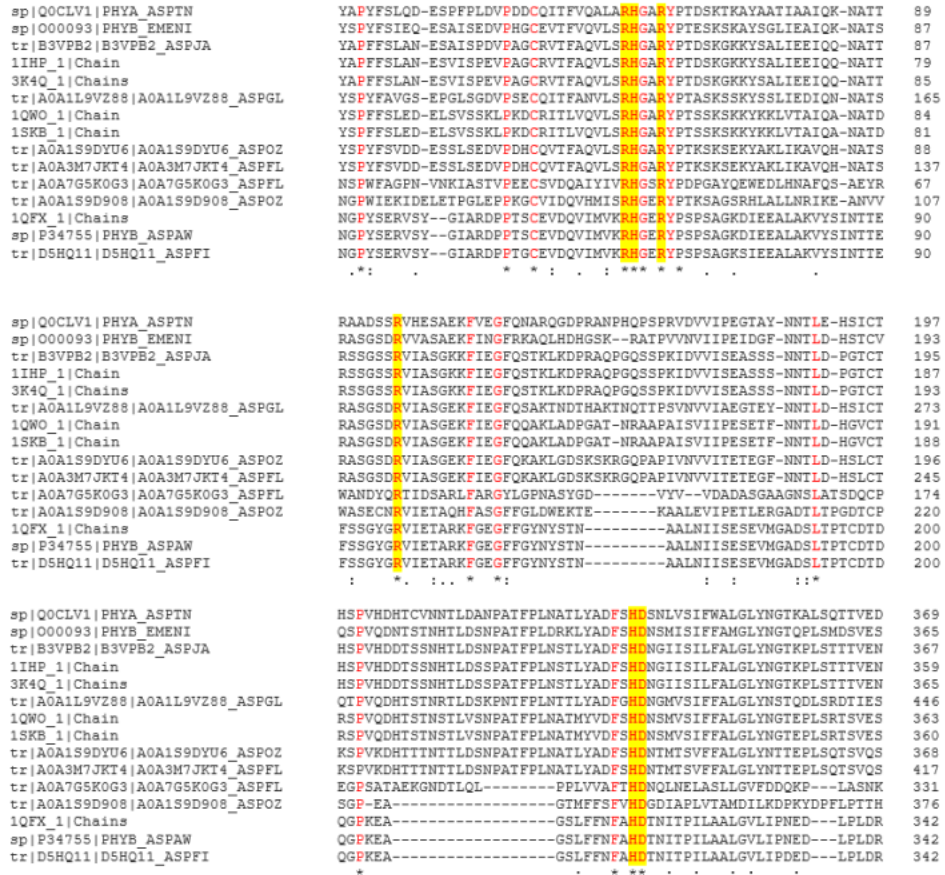
Figure 1. Multiple sequence alignment of 15 homologous phytase sequences
Gambar 1. Pensejajaran multisekuen dari 15 sekuen fitase yang homolog

Multiple sequence alignments of the 15 phytase sequences showed similarity (homology) in all sequences (Figure 1). The red letters indicate the conserved residues of *Aspergillus* phytase, while the yellow highlighted ones indicate the conserved residues of phytase from all living organisms. The catalytic site of the enzyme is the RHRRHD sequence motif consisting of residue Arg, His, Arg, Arg, His, and Asp. Substrate binding sites were identified in the form of homologous Asp and Glu residues in all sequences. Similar residues, known as conserved domains, are several residues gathered in one region and are shared in proteins with similar functions. These domains are found in proteins with similar functions even though they come from different species and generally share similar functional activities such as catalytic mechanisms. Conserved domains are a characteristic that may indicate the relationship of similar proteins from different organisms (Li et al., 2019). The conserved sequences in *Aspergillus* phytases play an important role as the main domain because they act as catalytic sites and provide the same catalytic activity in phytic acid hydrolysis (Wang et al., 2021).

Molecular phylogenetic analysis is a method that is used to determine the relationship among species through statistical comparison of their DNA sequences (Yu et al., 2017). In phylogenetic analysis, a phylogenetic tree is constructed to visualize the evolutionary relationship of sample organisms based on their sequence similarities (Tindi et al., 2017). Poisson model with pairwise deletion method is used to construct the phylogenetic tree. The goal of this model is to correctly identify trees at neighbor positions and produce branches that is as close to the original data as possible. A close relationship to the parent (ancestor) based on branch length, distance scale and node parameters is denoted by the branch length of 0.0, a small distance value, and a closer relationship between the parents. The gene sequences are combined to construct the predicted tree branches and calculate the branch length, which is the most accurate method for trees with short branches (Pangestika et al., 2015).

The phylogenetic analysis of 15 phytase protein sequences resulted in a rectangular phylogenetic tree with two main branches at the ancestral node (Figure 2). The first branch consists of 10 sequences and the
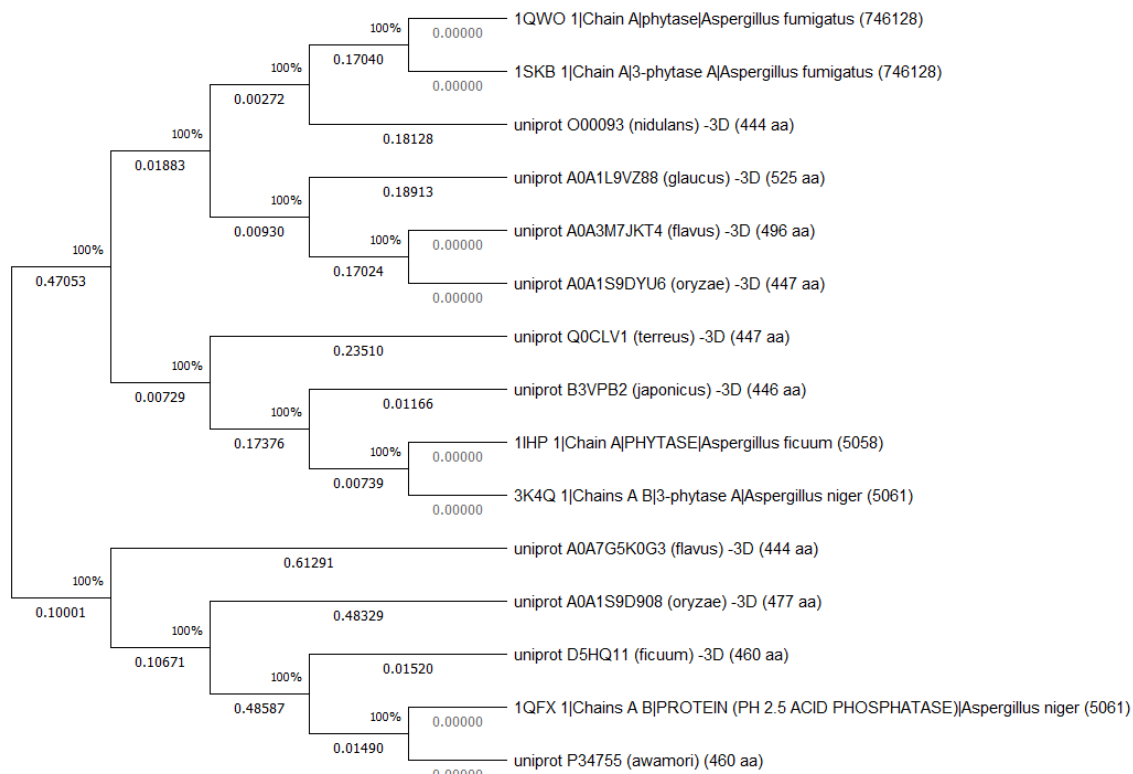
Figure 2 . Phylogenetic tree of 15 homologous *Aspergillus* phytases
Gambar 2. Pohon filogenetik 15 fitase dari Aspergillus *yang homolog*

second branch consists of 5 sequences. Each branch is divided into two nodes with a different number of sequences in each node. The phytase 1QFX has the closest relationship to P34755 with a difference in branch length values of 0.0. 1QFX also has a close relationship with phytase D5HQ11 with a difference in branch length values of 0.0003. This shows that there is a close relationship among phytases from *A. niger* phytase, *A. awamori*, and *A. ficuu*m. This result is similar to the homology analysis results, indicating the link between sequence similarity and the relationship of the enzyme source species.

Prediction of physicochemical characteristics

Physicochemical characteristics are typical characteristics of a protein determined by the arrangement, composition and conformation of its constituent amino acid residues (Bintang et al., 2020). The physicochemical characteristics of a protein influence its macroscopic physical appearance and molecular activity. These characteristics are the main parameters that determine the direction of production and application of a protein. Thus, determining the physicochemical characteristics is an important part in the selection and optimization processes of proteins

and enzymes. Some physicochemical characteristics of proteins that are commonly determined are isoelectric point, hydrophobicity, optical properties, density, solubility and toxicity (Gómez et al., 2017). Although some physicochemical characteristics can only be determined through experiments, most characteristics can be predicted by utilizing protein sequence alignment and physicochemical property prediction softwares (Kostrewa et al., 1999).

Predicted physicochemical parameters of 15 *Aspergillus* phytases using ProtParam showed different results (Table 2). However, it was observed that 1QFX had identical physicochemical characteristics to P34755 sequence. In addition, the properties of these two phytases are almost similar to D5HQ11. This result is consistent with the fact that the three phytases are very closely related and therefore have similar physicochemical characteristics. The highest number of residues was observed in phytase A0A1L9VZ88 (525 residues) while the lowest was observed in 1IHP (438 residues). In general, the number of residues is positively correlated with molecular weight. According to Bintang et al. (2020), the increase in protein molecular weight is an indication of good protein thermostability due to the

Table 2. Physicochemical characteristics of 15 phytase proteins
Tabel 2. Karakteristik fisikokimia dari 15 protein fitase

Protein accesion code *Kode aksesi protein*	Residue count *Jumlah residu*	Stability index *Indeks stabilitas*	pI *Titik isoelektrik*	Aliphatic index *Indeks alifatik*	GRAVY *GRAVY*	Asp+Glu (%) *Asp+Glu (%)*	Arg+Lys (%) *Arg+Lys (%)*
P34755	460	35.10	4.51	69.61	-0.340	50	26
1QFX	460	35.10	4.51	69.61	-0.340	50	26
D5HQ11	460	34.08	4.45	70.67	-0.315	54	26
A0A1S9D908	477	39.45	5.30	75.26	-0.389	60	43
A0A7G5K0G3	444	35.96	4.81	79.50	-0.314	49	34
1QWO	442	28.28	7.04	74.24	-0.260	45	45
1SKB	439	28.41	7.04	73.96	-0.263	45	45
3K4Q	444	45.41	4.94	72.25	-0.304	51	34
1IHP	438	43.90	4.88	73.01	-0.282	51	33
B3VPB2	446	43.86	4.94	72.38	-0.301	51	34
O00093	444	32.22	5.28	70.32	-0.353	52	38
A0A1S9DYU6	447	35.10	4.87	66.09	-0.412	57	39
A0A3M7JKT4	496	35.72	5.11	72.70	-0.310	60	46
Q0CLV1	447	36.56	5.12	70.38	-0.309	51	33
A0A1L9VZ88	525	34.24	4.96	65.60	-0.452	63	44

large number of amino acids that provide mutual interactions and chemical bonds to maintain protein integrity. It was observed that six proteins had quite large molecular weights, namely 1QFX, A0A1L9VZ88, A0A1S9D908, A0A3M7JKT4, D5HQ11 and P34755.

The stability index indicates the approximate stability of the protein *in vitro*. A protein with a stability index above 40 indicates that the protein is less stable. Based on the results, 1IHP, 3K4Q, and B3VPB2 are unstable due to their stability index being greater than 40. The aliphatic index is a number that indicates the volume of protein filled with long-chain amino acids. The aliphatic index cannot be used as a reference for determining protein parameters (Yu et al., 2017).

Isoelectric pH or pI is the pH at which a protein in a solution precipitates because it loses the ability to bind water (Azhari et al., 2017). pI generally does not have much difference from the optimum pH, therefore the ideal phytase for poultry feed should have a pI that ranges between 3.47 – 6.43 to adapt to the pH of animal's digestive tract. 1QWO and 1SKB are must be avoided because they have a pI of 7.04 that is in the weak base range. On the other hand, both 1QFX and P34755 had a pI of 4.51 while D5HQ11 had a pI of 4.45. These three phytases have a pI in the range of 3.47 – 6.43 and therefore are suitable for application in poultry feed.

GRAVY or grand average of hydropathicity is the sum of regular hydropathy of a protein. The GRAVY value of a protein is a measure of its hydrophobicity or hydrophilicity. The two measures are combined in a hydropathy scale or hydropathy index. Hydropathy values range from -2 to +2 for most proteins, with positively charged proteins being more hydrophobic (Kaur et al., 2020). The results showed that none of the 15 phytases tested has a GRAVY value above +1.

The structure of 1QFX, D5HQ11 and P34755

Structural modeling was carried out to predict the three-dimensional structure of phytases which empirical structures are not yet available in the RCSB PDB database. The modeling is carried out using the fold recognition principle to obtain more accurate results by recognizing domains and folds in the actual structure. AlphaFold is an AI system developed by DeepMind that predicts a protein's 3D structure from its amino acid sequence. AlphaFold greatly improves the accuracy of structure prediction by incorporating novel neural network architectures and training procedures based on the evolutionary, physical, and geometric constraints of protein structures (Jumper et al., 2021). The modeled phytase structure is the structure with the highest similarity and is suitable for the poultry digestive tract. 1QFX from *A. niger*, D5HQ11 from *A. ficuum*, and P34755 from *A. awamori* are the three structures that have the highest similarity, so it is necessary to know their protein structures for further analysis. The structure of 1QFX is available on the RCSB PDB website (Figure 3). Meanwhile, the structures of D5HQ11 and P34755 are not yet available and therefore need to be modeled using AlphaFold.

The 1QFX phytase is a homodimeric protein with two identical polypeptide chains, namely chains A and B. Each chain consists of 460 amino acid residues with several attached ligands such as N-acetylglucosamine and mannose, although these two ligands do not play

a role in the catalytic process. Therefore, it is necessary to remove these ligands in molecular docking analysis (Wang et al., 2021).

The modeled structure is different from the structure determined by direct crystallography. Modeled structures generally do not contain natural ligands because structural modeling programs only focus on amino acid sequences as a structure modeling template (Jumper et al., 2021). The structure of D5HQ11 modeled with AlphaFold consists of four homotetramer chains where each chain consists of 460 residues (Figure 4A). There is no natural ligand attached to the modeled structure. A similar result was also observed in the P34755 modeled structure (Figure 4B).

The three structures were then compared using a PyMOL. Superpose was conducted on 1QFX,

D5HQ11 and P34755 (Figure 5). The structure comparison results show that the three proteins have a high similarity on each domain, although they have small differences in physicochemical characters. The active site residues were also shown on the same position as it was shown on the result. The parameters used to determine the protein quality and homology was the RMSD or root mean square deviation. This value is the result of the comparison atom positions in residues that make up the protein structure of 1QFX, D5HQ11 and P3755. A good RMSD is smaller than 2 Å as it shows a high compatibility among atoms in the protein structures (Wang et al., 2021). The three structures have an RMSD value of 0.557 Å, indicating a substantial similarity.
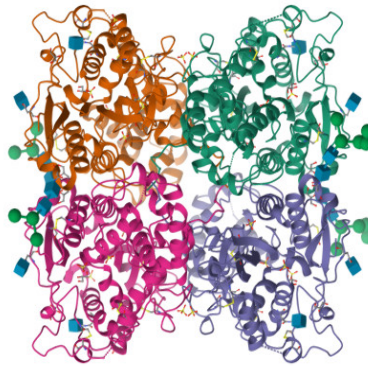
Figure 3. 3D structure of phytase 1QFX from RCSB PDB
Gambar 3. Struktur fitase 1QFX dari RCSB PDB

A   B
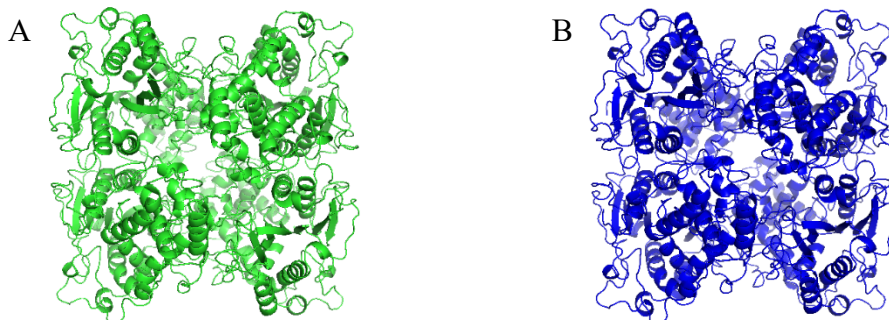
Figure 4. 3D structures of phytase (A) D5HQ11 and (B) P34755 modeled with AlphaFold
Gambar 4. Struktur (A) D5HQ11 dan (B) P34755 hasil pemodelan dengan AlphaFold
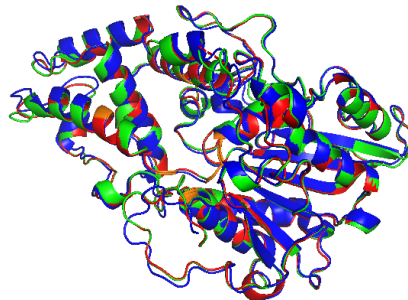
Figure 5.   Superpose of 1QFX (shown in red), D5HQ11 (green) and P34755 (blue). Active sites resides are marked in orange
Gambar 5. Superpose dari 1QFX (merah), D5HQ11 (hijau) dan P34755 (biru). Warna jingga menunjukkan residu situs aktif

Structure quality of 1QFX, D5HQ11 and P34755

The structural quality of the protein modeled with AlphaFold can be determined using the Ramachandran plot. Ramachandran plot is a diagram constructed from the distribution of amino acid residues at two types of angles; the phi (ϕ) in the x-axis and the psi (ψ) in the y-axis (Schmidt et al., 2013). The Ramachandran plot is divided into four quadrants: the most favored regions (quadrant I) in red, additional allowed regions (quadrant II) in yellow, generously allowed regions (quadrant III) in pale yellow, and disallowed regions (quadrant IV) in white. While the Ramachandran plot for D5HQ11 and P34755 were modeled using Procheck, the Ramachandran plot of 1QFX can be downloaded from RCSB PDB.

The analysis results showed that 1QFX had 90.3% residues in quadrant I, 9.2% residues in quadrant II, 0.5% residues in quadrant III, and 0% residues in quadrant IV (Figure 6A). The D5HQ11 had 89.8% residues in quadrant I, 9.6% residues in quadrant II, 0.3% residues in quadrant III, and 0.3% residues in quadrant IV (Figure 6B). Meanwhile, the Ramachandran plot of P34755 shows that this enzyme had 89.8% residues in quadrant I, 9.7% residues in quadrant II, 0.4% residues in quadrant III, and 0% residues in quadrant IV (Figure 6C). A good quality model or structure is expected to have more than 80% residues in the most favored region and less than 1% non-glycine residues in disallowed regions (Peter et al., 2017). The 3D structure of 1QFX obtained from RCSB PDB was a good structure. The 3D models of D5HQ11 and P34755 also have a good quality because there are more than 80% residues in the most favored region.

Visualization of molecular docking

Molecular docking was carried out using phytic acid (CID 890) attached to 1QFX, D5HQ11, and P34755. The molecular docking carried out in this study is a targeted docking in which the test ligand or substrate is directly attached to the active site of the enzyme (Figure 7). This is because the modeled protein does not have a natural ligand. The gridbox creation and test ligand docking were carried out directly on the active site known from previous analyses (Suhadi et al., 2013). Active site residues in 1QFX are Arg62, His63, Arg66, Asp75, Arg156, Glu272, His318 and Asp319. Residues Asp75 and Glu272 act as substrate specificity sites, while other residues act as catalytic sites (Wang et al., 2021). These residues are conserved and have the same position and role in D5HQ11 and P34755 enzymes.

Molecular docking method is based on the formation of a complex molecular conformational structure formed in ligand or substrate and protein interaction. The stability of the resulting complex molecule conformation was then analyzed (Meyer et al., 1998). This study used YASARA Structure as the docking software because it has the advantage of having an energy minimization feature. The docking parameters that can be analyzed with YASARA include the affinity energy and dissociation constant. A good docking results in protein-ligand binding with a high affinity energy and low dissociation constant (Agistia et al., 2013).

The docking results in the form of binding points for the phytic acid substrate on each residue are then compared with the active site of the phytase enzyme (Table 3). The docking analysis results show that 1QFX has a substrate binding site that is most identical to the active site of the protein with 6 binding points. D5HQ11 has a binding point of 4 residues that are identical to the active site. P34755 only has 3 identical active sites. The comparison results also show that the residue Arg62 and Arg66 have a substrate binding function in all three enzymes. This is because Arg62 and Arg66 are conserved catalytic residues in phytase (Kostrewa et al., 1999).
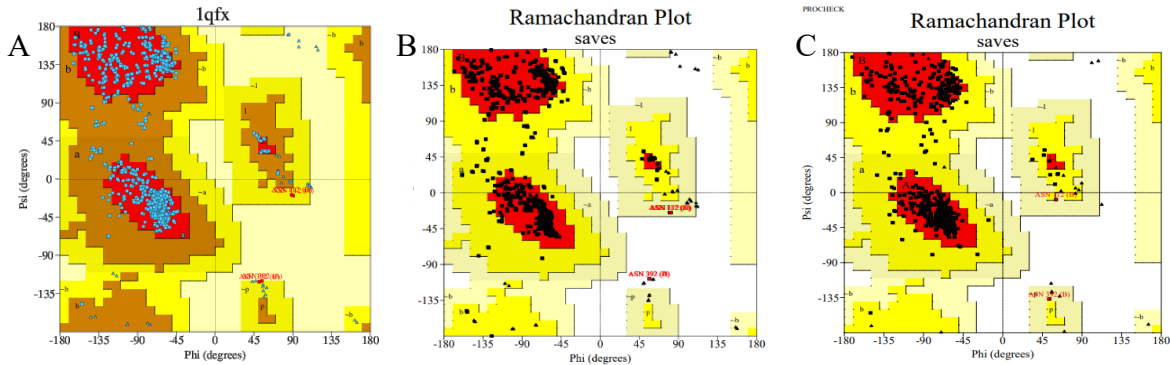
Figure 6. Ramachandran plot of phytase structure (A) 1QFX, (B) D5HQ11, (C) P34755
Gambar 6. Plot Ramachandran dari struktur fitase (A) 1QFX, (B) D5HQ11, (C) P34755
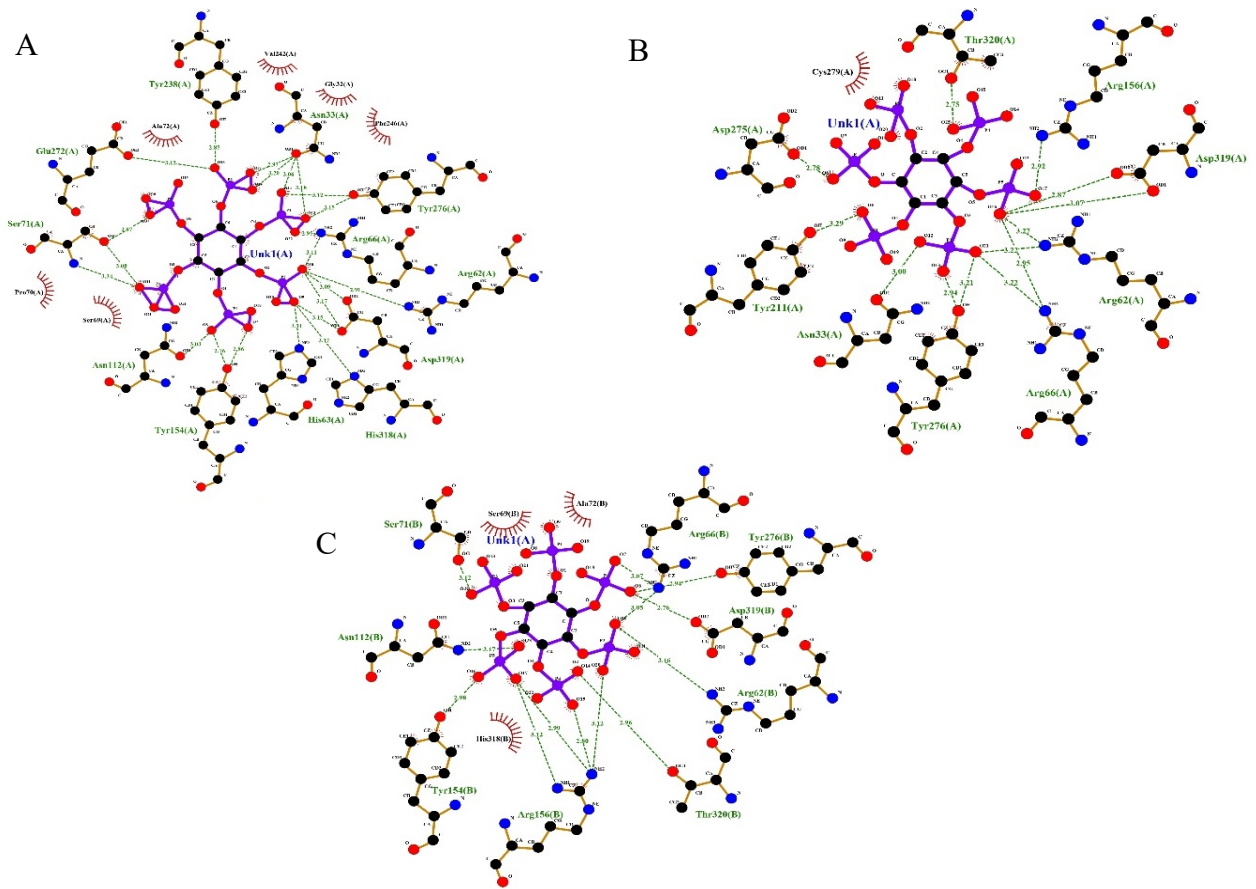

Figure 7. 2D visualization of the active site of phytase (A)1QFX, (B) D5HQ11, and (C) P34755 with phytic acid as a ligand
Gambar 7. Visualisasi 2D sisi aktif pengikatan ligan asam fitat dengan fitase (A)1QFX, (B) D5HQ11, dan (C) P34755

Visualization of the docking results using PyMOL shows the binding pocket that binds to phytic acid in each enzyme. The phytase enzyme has a long pocket with 2 small holes at the entrance and a narrow middle part. The phytic acid molecule is shown as a stick structure (Figure 8). Based on the visualization results, the binding pocket is similar for each enzyme, especially at the catalytic site. The catalytic sites were far from Asp75 and Glu272, the substrate specificity residues. All ligands are visible on the surface of the binding pocket in all enzymes.

Energy affinity and dissociation constant

The result of molecular docking is a log file containing affinity energy and dissociation constant values (Table 4). The binding energy indicates the interaction strength between a protein and its ligand in the form of affinity energy (ΔG). A lower affinity energy indicates a spontaneous binding process that forms a stable and strong bond between the ligand and protein. Conversely, the greater the affinity energy, the more unstable the conformation and bonding are (Abusham et al., 2019). On the other hand, dissociation constant (Kd) quantifies the equilibrium between a ligand being free in solution and bound to the protein. The smaller constant indicates high substrate affinity to protein (Erna et al., 2016).

Table 3. Comparison of each docked protein interactions
Tabel 3. Perbandingan interaksi tiap reseptor yang ditambatkan

Proteins *Protein*	Amino acid residue *Residu asam amino*	
	Hydrogen bond *Ikatan hidrogen*	Hydrophobic bond *Ikatan hidrofobik*
1QFX	Asn33, **Arg62**, **His63**, **Arg66**, Ser71, Asn112, Tyr154, Tyr238, **Glu272**, Tyr276, **His318**, **Asp319**	Gly32, Ser69, Pro70, Ala72, Val242, Phe246
D5HQ11	Asn33, **Arg62**, **Arg66**, **Arg156**, Tyr211, Asp275, Tyr276, **Asp319**, Thr320	Cys279
P34755	**Arg62**, **Arg66**, Ser71	Ser69, Ala72, **His318**

Note: Residues in bold are shared between the docked proteins and the known active site residues from each enzyme
Keterangan: *Residu bercetak tebal merupakan residu yang sama antara residu hasil penambatan dengan situs aktif masing-masing enzim*
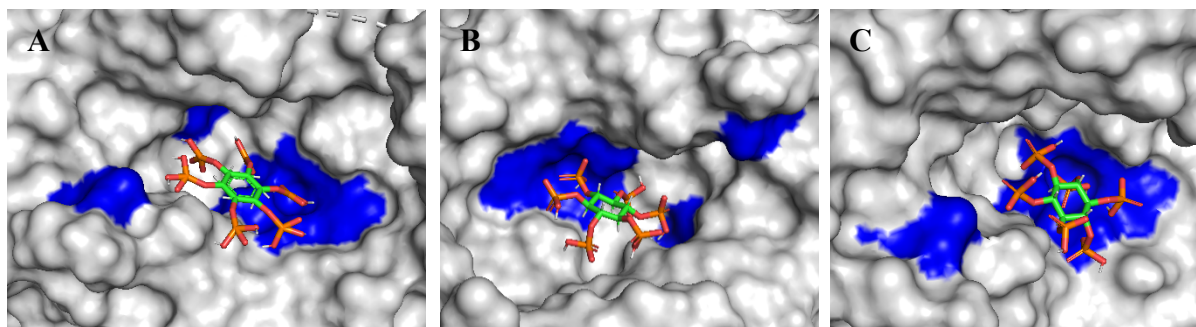

Figure 8.   Visualization of phytic acid (orange and green sticks) bound to the binding pockets of phytase (A) 1QFX, (B) D5HQ11, and (C) P34755. Enzyme active sites are colored in blue
Gambar 8. Visualisasi asam fitat dalam binding pocket *enzim fitase (A) 1QFX, (B) D5HQ11, dan (C) P34755. Warna biru menunjukkan situs aktif masing-masing enzim*

Table 4. Molecular docking interactions of phytase
Tabel 4. Interaksi penambatan molekuler fitase

Ligand *Ligan*	Enzyme *Enzim*	Affinity energy (ΔG) (kcal/mol) *Energi afinitas (ΔG) (kkal/mol)*	Dissociation constant (Kd) (µM) *Konstanta disosiasi (Kd) (µM)*
Phytic acid	1QFX	-7.99	1.3940
	D5HQ11	-6.80	10.3299
	P34755	-6.46	18.3263

The affinity energy of 1QFX, D5HQ11, and P34755 enzyme are -7.99 kcal/mol, -6.8 kcal/mol, and -6.46 kcal/mol, respectively. 1QFX has the most negative affinity energy and therefore it has the strongest affinity to phytic acid. Similar results were also shown for the Kd constant with the results for 1QFX, D5HQ11, and P34755 enzyme being 1.3940 μM, 10.3299 μM, and 18.3263 μM, respectively. 1QFX has the lowest Kd value means that it has the strongest affinity to the phytic acid (Dermawan et al., 2013). In summary, phytase 1QFX from *A. niger* has the strongest affinity and binding to phytic acid.

Phytase has been used in animal nutrition for two decades as a sustainable mineral source and became a standard ingredient in feed for monogastric animals. In swine and poultry, phytase addition has had positive impacts on the availability of minerals such as phosphorus, calcium, and zinc. Phytase addition also reduces the fecal excretion of phosphorus. Phytases are added to around 70% of the monogastric animal diet (Troesch et al., 2013). In human studies, the use of phytase to date has mainly focused on the benefit of iron and zinc absorption. *A. niger* provided the very first industrial phytase, which was categorized as a 3-phytase (Ningsih et al., 2017). This research was focused at in silico analyses of phytases. In silico protein analysis is uses computational tools to simplify analysis that previously had to be carried out using more complicated steps. The analyses are generally carried out as an initial screening step to determine the protein structure characteristics to be studied further. The use of computational tools will produce data to support laboratory experiments related to protein characterization (Madden et al., 2020).

Conclusion

Phytase enzymes with original accession code 1QFX from *A. niger*, D5HQ11 from *A. ficuum*, and P34755 from *A. awamori* have a fairly high level of homology and structural similarity. The two enzymes are also similar in terms of phylogeny, have a relatively small evolutionary distance, and similar physicochemical parameters. The docking results in the form of ΔG and Kd show that 1QFX has the strongest affinity and binding with its substrate phytic acid. In conclusion, the phytase enzyme from *A. niger* is the most suitable for production and application in poultry feed.

References

Abusham, R. A. K., Masomian, M., Salleh, A. B., Leow, A. T. C. & Rahman, R. N. Z. (2019). An in-silico approach to understanding the structure-function: a molecular dynamics simulation study of rand serine protease properties from *Bacillus subtilis* in aqueous solvents. *Advances in Biotechnology & Microbiology*, *12*(1), 9-17.

Agistia, D. D., Purnomo, H., Tegar, M. & Nugroho, A. E. (2013). Interaksi senyawa aktif dari *Aegle marmelos* L. (Correa) sebagai anti inflamasi dengan reseptor COX-1 dan COX-2. *Traditional Medicine Journal*, *18*(2), 80-87.

Akdel, M., Durairaj, J., de Ridder, D. & van Dijk, A. D. J. (2020). Caretta – a multiple protein structure alignment and feature extractionsuite. *Computational and Structural Biotechnology Journal, 18*, 981–992. https://doi.org/10.1016/j.csbj.2020.03.011

Azhari, A., Jalaludin, D. & Irawan, A. (2017). Proteomik: database dan teknologi. *Quantum: Jurnal Inovasi Pendidikan Sains*, *8*(1), 1-12.

Bintang, M., Rahmawati, F., Safira, U. M. & Andrianto, D. (2020). *Biokimia fisik*. IPB Press.

Coban, H. B. & Demirci, A. (2017). *Handbook of food bioengineering*. Academic Press.

Cominelli, E., Pilu, R. & Sparvoli, F. (2020). Phytic acid and transporters: what can we learn from low phytic acid mutants. *Plants*, *9*(69), 1-20.

Daugelaite, J., O'Driscoll, A. & Sleator, R. D. (2013). An overview of multiple sequence alignments and cloud computing in bioinformatics. *ISRN Biomathematics*, 2013, *1*-14. http://dx.doi.org/10.1155/2013/615630

Dermawan, D., Sumirtanurdin, R. & Dewantisari, D. (2019). Simulasi dinamika molekular reseptor estrogen alfa dengan andrografolid sebagai anti kanker payudara. *IJPST*, *6*(2), 65-76.

Erna, M., Emriadi, Alif A. & Arief, S. (2016). Sifat termodinamika dan karakterisasi inhbisi korosi nano-partikel kitosan pada permukaaan baja lunak dalam media air gambut. *Alchemy*, *12*(1), 27-35.

Gómez, D. C. N., Hoyos, C. M. R., Álvarez, E. D. M., Montaño, E. A. R., Alejo, N. E. V., Casallas, I. N. R., Türkmen, K. E., Suárez, H. S., Moreno, J. A. S., Piñales, R. A. P., Santos, J. G. & Galvis, A. A. (2017). In silico characterization of 3-Phytase A

and 3-Phytase B from *Aspergillus niger*. *Enzyme Research*, 2017, 1-23. https://doi.org/10.1155/2017/9746191

Irfanuddin (2018). Karakteristik biomolekuler mutasi amyloid precursor protein sebagai penyebab penyakit alzheimer yang dianalisis melalui media bioinformatika. Sriwijaya *Journal of Medicine*, *1*(1), 61-73.

Jumper, J., Evans, R., Pritzel, A. *et al.* (2021). Highly accurated protein structure predicyion with AlphaFold. *Nature*, *596*, 583-589. https://doi.org/10.1038/s41586-021-03819-2

Karunasekera, H. (2013). Molecular identification and phylogenic analysis by sequencing the rDNA of copper-tolerant soft-rot *Phialophora* spp. *International Biodeterioration and Biodegradation*, *82*, 45-52.

Kaur, A., Pati, P. K., Pati, A. M. & Nangpal, A. K. (2020). Physico-chemical characterization and topological analysis of pathogenesis-related proteins from *Arabidopsis thaliana* and *Oryza sativa* using in-silico approaches. *PLoS ONE*, *15*(9), e0239836. https://doi.org/10.1371/journal.pone.0239836

Kostrewa, D., Wyss, M., D'Arcy, A. & Loon, A. P. G. M. V. (1999). Crystal structure of *Aspergillus niger* pH 2.5 acid phosphatase at 2.4 Å resolution. *Journal of Molecular Biology*, *288*(5), 965-974. https://doi:10.1006/jmbi.1999.2736

Lei, X. G., Weaver, J. D., Mullaney, E. & Ullah, A. H., Azain, M. J. (2013). Phytase, a new life for an "old" enzyme. *Reviews in Advance*, *1*(1), 1-27. htpps://doi.org./10.1146/annurevanimal-031412-103717

Li, Y. C. & Lu, Y. C. (2019). BLASTP-ACC: parallel architecture and hardware accelerator design for blast-based protein sequence alignment. *IEEE Transactions on Biomedical Circuits and Systems*, *13*(6), 1771-1782.

Madden, J. C., Enoch, S. J., Paini, A. & Cronin, M. T. D. (2020). A review of in silico tools as alternatives to animal testing: principles, resources and applications. *Alternatives to Laboratory Animals*, *48*(4), 146–172. http://dx.doi.org/10.1177/026119292096597

Meyer, M., Wohlfahrt, G., Knäblein, J. & Schomburg, D. (1998). Aspects of the mechanism of catalysis of glucose oxidase: A docking, molecular mechanics and quantum chemical study. *Journal of Computer-Aided Molecular Design*, *12*(5), 425-440. https://doi.org/10.1023/A:1008020124326

Mizana, D. K., Suharti, N. & Amir, A. (2016). Identifikasi pertumbuhan jamur *Aspergillus* sp pada roti tawar yang dijual di Kota Padang berdasarkan suhu dan lama penyimpanan. *Jurnal Kesehatan Andalas*, *5*(2), 355-360.

Ningsih, D. A., Kusdiyantini, E. & Raharjo, B. (2017). Uji aktivitas enzim fitase yang dihasilkan oleh *Aspergillus niger* dan *Neurospora* sp. pada kondisi fermentasi yang berbeda. *Jurnal Biologi*, *6*(4), 19-28.

Nurhikmah. (2017). *Isolasi dan skreening bakteri endofit penghasil enzim fitase dari tanaman jagung* (*Zea mays*). [SKRIPSI] Departemen Biologi, Universitas Islam Negeri Alauddin, Makassar.

Pangestika, Y., Budiharjo, A. & Kusumaningrum, H. P. (2015). Analisis filogenetik *Curcuma zedoaria* (temu putih) berdasarkan gen internal transcribed spacer (ITS). *Jurnal Biologi*, *4*(4), 8-13.

Peter, I., Maxwell, Paul & L. A. Popelier. (2017). Unfavorable regions in the ramachandran plot: Is it really steric hindrance? The interacting quantum atoms perspective. *Journal of Computational Chemistry*, *38*(29), 2459-2474. https://doi.org/10.1002/jcc.24904

Pramanik, K., Pal, P., Soren, T., Mitra, S., Ghosh, P. K., Sarkar, A. & Maiti, T. S. (2018). In silico structural, functional and phylogenetic analysis of *Klebsiella* phytases. *Journal of Plant Biochemistry and Biotechnology*, *5*(1), 362-372. https://doi.org/10.1007/s13562-018-0445-y

Pratiwi, N.W.K., El Auly, F., Amrulloh, R. & Kurniawati, F. (2020). Deteksi dan identifikasi nematoda puru akar (*Meloidogyne* spp.) pada tanaman bit menggunakan metode DNA barcoding. *Jurnal Fitopatologi Indonesia*, *16*(1), 1-8. https://doi.org/10.14692/jfi.16.1.1–8

Purnamasari L & Miswar (2018). Phytase enzyme characteristic of *Bacillus subtilis* to increase the quality of poultry feed. *Journal of Livestock Science and Production*, *2*(2), 93-98.

Ramdani ED, Yanuar A & Tjandrawinata RR (2019). Comparison of dopamine D2 receptor (homology model and X-ray structure) and virtual screening protocol validation for the antagonism mechanism. *Journal of Applied Pharmaceutical Science*, *9*(9), 17-22. https://doi.org/10.7324/JAPS.2019.90903

Rasyid, E.K.P.(2017). *Tingkat hidrolisis asam fitat pada berbagai pakan broiler dengan penambahan fitase bakteri endofit asal tanaman jagung (Zea mays) secara in vitro*. [Skripsi] Departemen Biologi, Universitas Islam Negeri Alauddin.

Schmidt, T., Bergner, A. & Schwede, T. (2013). Modeling three-dimensional protein structures for applications in drug design. *Drug Discovery Today*, *19*(7), 890- 897.

Silvia, E. O. & Bracarense, A. P. F. R. L. (2016). Phytic acid: from antinutritional to multiple protection factor of organic systems. *Journal of Food Science*, *81*(6), 1357- 1362. https://doi.org/10.1111/1750-3841.13320.

Suhadi, A., Rizarullah, R. & Feriyani. (2019) Simulasi docking senyawa aktif daun binahong sebagai inhibitor enzim *aldose reductase*. *Jurnal Penelitian Kesehatan*, *6*(2), 55-65. http://dx.doi.org/10.22435/sel.v6i2.1651

Syaifurrisal, A. (2014). *Pengaruh penyimpanan pakan udang komersial dengan penambahan volume air berbeda terhadap pertumbuhan jamur dan kandungan protein kasar*. [Skripsi] Departemen Kimia, Universitas Airlangga.

Tindi, M., Mamangkey, N.G. & Wullur, S. (2017). DNA barcode dan analisis filogenetik molekuler beberapa jenis bivalvia asal perairan Sulawesi berdasarkan gen COI. *Jurnal Pesisir dan Laut Tropis*, *1*(2), 32-38.

Troesch, B., Jing, H., Laillou, A. & Fowler, A. (2013). Absorption studies show that phytase from *Aspergillus niger* significantly increases iron and zinc bioavailability from phytate-rich foods. *Food and Nutrition Bulletin*, *34*(2), 90-101. https://doi.org/10.1177/15648265130342S111

Wang, Y., Zhang, H., Zhong, H. & Zhidong, X. (2021). Protein domain identification methods and online resources. *Computational and Structural Biotechnology Journal*, *19*, 1145–1156. https://doi.org/10.1016/j.csbj.2021.01.041

Widodo, T. S., Sulistiyano, B. & Utama, C. S. (2015). Jumlah bakteri asam laktat (BAL) dalam digesta usus halus dan sekum ayam broiler yang diberi pakan ceceran pabrik pakan yang difermentasi. *Agripet*, *15*(2), 98 – 103. http://dx.doi.org/10.17969/agripet.v15i2.2376

Yanuartono, Nururrozi, A., Indarjulianto, S., Purnamaningsih, H. & Raharjo, S. (2019). Metode tradisional pengolahan bahan pakan untuk menurunkan kandungan faktor antinutrisi: review singkat. *Jurnal Ilmu Ternak*, *19*(2), 97-107. http://dx.doi.org/10.24198/jit.v19i2.23974

Yu, L., Zhang, Y., Gutman, I., Shi, Y. & Dehmer, M. (2017). Protein sequence comparison based on physicochemical characteristics and the position-feature energy matrix. *Scientific Reports*. *7*(46787), 1-8. https://doi.org/10.1038/srep46787

Yuan, S., Chan, S. & Hu, Z. (2017). Using PyMOL as a platform for computational drug design. *Wiley Interdisciplinary Reviews: Computational Molecular Science*, *7*(2), 1-10 https://doi.org/10.1002/wcms.1298

Zahara, N., Soekarno, B. P. W. & Munif, A. (2018). Metabolit bakteri endofit asal tanaman kacang tanah sebagai penghambat pertumbuhan *Aspergillus flavus*. *Jurnal Fitopatologi Indonesia*, *14*(1), 15-22. https://doi.org/10.14692/jfi.14.1.15