

Analysis to describe the catalytic critical residue of keratinase mojavensis using peptidase inhibitors: A docking-based bioinformatics study

Somayyeh Rahimnahal^{1,2}, Morteza Shams³, Hossein Tarrahimofrad^{2,4}, Yahya Mohammadi^{1*}

1. Department of Animal Science, Ilam University, Ilam, Iran
2. Department of Animal Science and Food Technology, Agriculture Science and Natural Resources University Khouzestan, Ahwaz, Iran
3. Zoonotic Diseases Research Center, Ilam University of Medical Sciences, Ilam, Iran
4. Department of Animal Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

*Corresponding author: Tel: +98 8432227015 Fax:-

Address: Department of Animal Science, Ilam University, Ilam, Iran

E-mail: mohamadi_yahya@yahoo.com

Received: 6/03/20 Revised: 28/04/2020 Accepted: 23/06/2020

Abstract

Introduction: Vital catalysts have long been widely used in the food industry, but with new applications in many industries, such as the chemical industry, they have become even more important. In biocatalysis, all parts of the cell, cell extract, purified enzyme, inactive cell, or inactive enzymes are used as catalysts in various processes. Enzymes are essential for various industrial, pharmaceutical, and especially biotechnological processes. Keratinase is produced by various microorganisms in the presence of keratin as a substrate. It mainly targets disulfide bonds. In this study, the biochemical properties of keratinase enzymes derived from *Bacillus mojavensis* (*B. mojavensis*) were investigated.

Materials and methods: The 3D structure of keratinases from *B. mojavensis* was created using Modeler software, and the model's validation and refinement indicators, including Prosa, Z-score, and Ramachandran Graph confirmed the high quality of the modeled protein. The PMSF, Pepstatin and leupeptin structures were prepared from the PubChem database server and introduced to the MVD software along with the 3D structure of the keratinase for molecular docking.

Results: The binding energies (Eaint#) for the Mojavensis-PMSF, Mojavensis-Pepstatin and Mojavensis-Leupeptin complexes were -71.73, -334.1 and -211.2, respectively. In all three Mojavensis-PMSF, Mojavensis-Pepstatin and Mojavensis-Leupeptin complexes, the Serine 277 keratinase mojavensis formed a hydrogen bond with inhibitors. Serine 295 also interacted with inhibitors in both of Pepstatin and Leupeptin complexes. Glutamic 299 keratinase mojavensis also interacted with PMSF and Leupeptin. All three PMSF, Pepstatin and leupeptin peptidase inhibitors were able to interact with keratinase mojavensis.

Conclusion: Docking results showed that Serine amino acids 277 and 295 in the active site of keratinase mojavensis, may play a key role in its catalytic function.

Keywords: Bioinformatics, Keratinase, Enzyme, Homology modeling, Docking

Introduction

A major constraint in many developing countries has been the steady rise in the price of feed components. As a result, cheaper and more common nutrients that

contain a large amount of non-starch (soluble and insoluble) polysaccharides with starch are used in animal nutrition in these countries (1). The most specific bird trait is

Copyright © 2020 Journal of Basic Research in Medical Science. This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>) which permits copy and redistribute the material, in any medium or format, provided the original work is properly cited.

having a feather. Feather powder is currently used to feed livestock and due to its high nitrogen content, feather is used to produce fertilizer in agricultural land (2). It is composed of 90 to 92% protein (keratin) and 1 to 8% fat (3). Keratin is a non-soluble structural protein that found in large scale in feathers, wool and hair of animals. Each of their filament-matrix units is bound together by a large number of disulfide bands, making it resistant to many physical, chemical and biological agents, thus becoming an ideal protective layer for animals. Based on its secondary structure, it is divided into two types of alpha and beta keratin (4). The use of enzymes has increased as an additive in animal nutrition rapidly. In the past decade, many studies have been done to investigate the effect of exogenous enzymes on poultry yield. By examining these studies, can conclude that the enzyme is an important tool for use in poultry feed (5). Keratinase is one of the proteolytic enzymes in nature. This enzyme is classified as a proteinase by an unknown mechanism with have E.C. 3.4.99.11 (6, 7). Keratinase is called a serine protease because 97% of its sequence is similar to alkaline protease and inhibited by serine protease inhibitors. And sometimes, it has been found as a serine protease in combination with a cysteine protease and a metalloprotease (8). keratinase produced by microorganisms has many applications in nutrition, fertilizer, in the detergent and leather industries, in pharmaceuticals and in fermentation technology (9). To further understand the properties of enzymes, the third structure of proteins, including enzymes, is determined using crystallographic methods. This method is very accurate but it requires a lot of time and cost (10). Using enzyme structure prediction tools is a cost-effective and efficient method and can lead to their selection and proper use. In this study, we investigated the

theoretical and structural biochemical properties of a mesophilic Mojavensis keratinase (Q6BCN9) using bioinformatics prediction tools and methods.

Materials and methods

Primary structure

The biochemical properties of the protein were investigated using the protparam program (<http://www.expasy.org/tools/protpar-ref.html>) (11). The Peptide signal was tested using the program SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) (<http://www.cbs.dtu.dk/services/SignalP>) (12). Using the website <http://disulfind.dsi.unifi.it>, the existence of a disulfide bond was investigated. (13).

Secondary structure prediction

The second structure of the protein was predicted using the online program <http://www.sbg.bio.ic.ac.uk/pHyre2>. (14). This server uses the self-optimized prediction method (SOPM) to increase the second-order forecast improvement rate based on the amino acid sequence.

Active site pocket prediction

The active site of the enzyme was predicted using two online applications. Initially the active amino acids of Keratinase mojavensis were predicted using the <http://www.sbg.bio.ic.ac.uk/~3dligandsite/>. (15). With the help of the online website <https://zhanglab.ccmb.med.umich.edu/COF-ACTOR/>, the binding sites of keratinase mojavensis were identified. (16, 17). Then, according to the results of both online software, amino acids of the active site were predicted.

Conserved sequence identification

Using the Uniprot database, the amino acid sequence obtained from other proteins

similar to keratinase *mojavensis* (with more than 95% similarity) was obtained and stored. The conserved sequence of Keratinase *mojavensis* was evaluated and compared using the Multiple Alignment Tool, www.ebi.ac.uk, based on the CLUSTAL O (1.2.4) algorithm.

Homology modeling

The 3D structure of the protein was simulated using Modeller software. Because the crystallographic structure of Keratinase *mojavensis* is not available, for this purpose, the amino acid sequence of Keratinase *mojavensis* was incorporated in Blast-PDB and from the crystallographic structure of 4GI3A (RCSB: 10.2210/pdb4GI3/pdb) that showed the most similarity and e-Value to Keratinase *mojavensis*.

Validation

The <http://services.mbi.ucla.edu/PROCHECK> and <http://services.mbi.ucla.edu/SAVES> was used for ensuring the accuracy of the simulated building. (18). Data from WHAT_CHECK, ERRAT, VERIFY_3D and CRYST1 record matches were also analyzed. Using the website <https://prosa.services.came.sbg.ac.at/prosa.pHp>, the Z-Score point and the protein energy balance was determined (19, 20). By using the online website <http://mordred.bioc.cam.ac.uk/~rapper/rampage.pHp> the Ramachandran Plot graph was drawn (21). Chimera V1.5.3 software determined the best spatial resolution for the optimal energy used in this study (22).

Ligand-protein molecular docking

PMSF, Pepstatin and leupeptin structures were prepared and stored from the PubChem database. Then the 3D keratinase structure was introduced to the MVD software. Using the minimize sidechain step, the energy of all the structures was optimized and the

water molecules around it were removed. Then cavities were predicted and identified on the keratinase structure. Each of the inhibitors was docked separately with the keratinase structure. For each docking complex, 10 runs were performed. Finally, the best complex was chosen for each docking ligand-protein that had the least amount of binding energy. The 2D results for each complex were showed by LigPlot⁺.

Results

Primary structure

Based on the information obtained from bioinformatics databases, keratinases are considered as a serine protease family. In general, keratinases have a second with the "dyad" or "tetrad" Serine-Histidine catalyst in the active site, which is nucleophilic attack by the serine amino acid present in the amino acid sequence.

Bioinformatics studies were performed to determine some of the biochemical properties of the enzyme, to investigate the presence of the peptide signal, and to investigate the formation of a disulfide bond. The enzyme had a molecular weight of about 38.86 kDa.

Based on the bioinformatics studies performed, the isoelectric point, the instability index and the net protein load of the Grand average of hydropathicity (GRAVY) are 8.73, 12.83 and -0.039, respectively. The aliphatic coefficient of the enzyme, which represents the volume occupied by the side chain of the amino acids ALA, Val, Leu, and Ile in the protein, was determined to be 83.93.

The half-life of a protein is the time it takes for half of the protein produced to be depleted, which is estimated to be the half-life of the protein given the number of amino acids in the N-terminal. In this study, keratinase *mojavensis* had 4 disulfide bonds.

Figure 1. The secondary structure of Keratinase mojavensis. α -helix is blue and β -sheet is green.

Conserved sequence identification

Amino acid sequence alignment of Keratinase mojavisensis was performed using

Blast at Uniprot database. The alignment results of the Keratinase mojavisensis sequence and the conserved regions are shown in Figure 2.

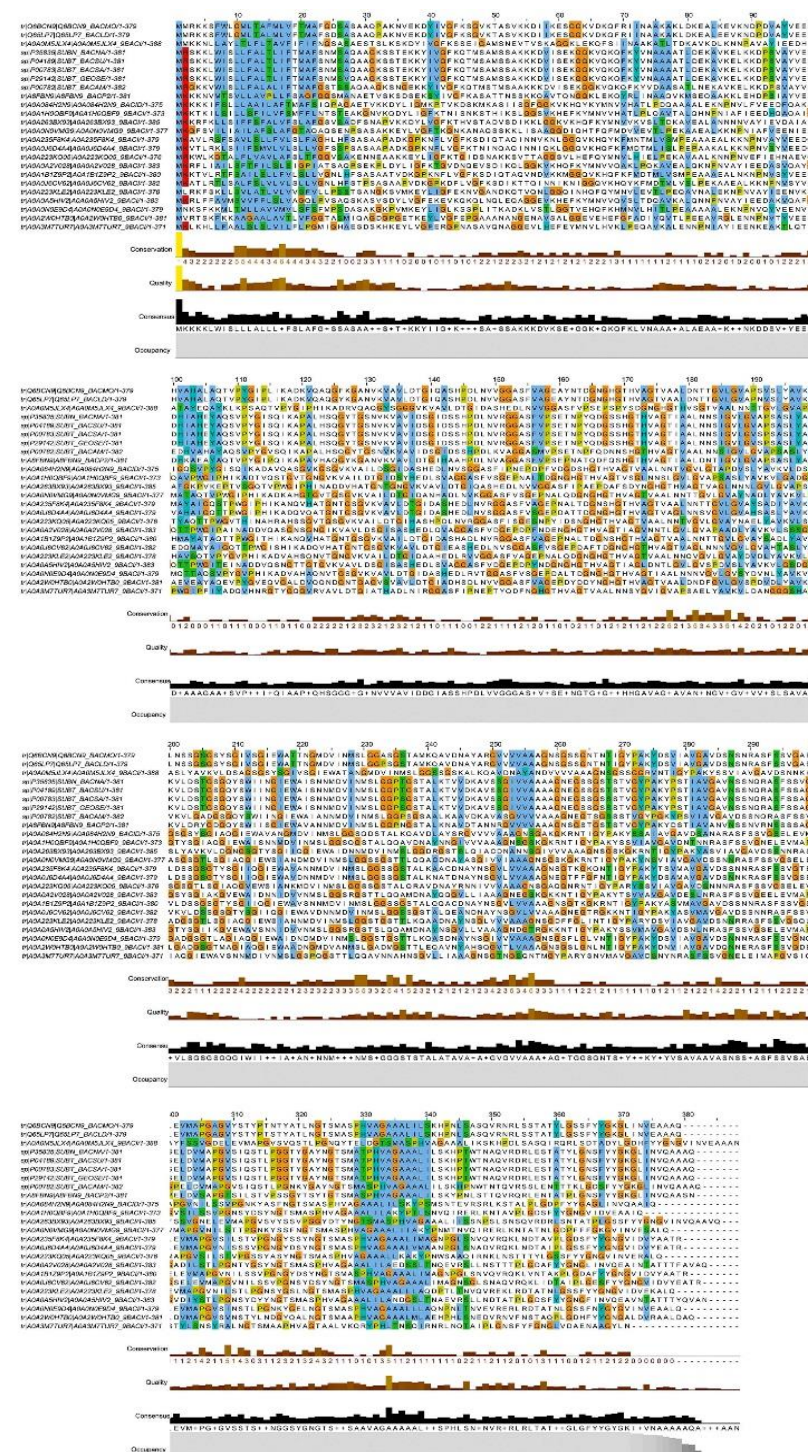


Figure 2. Conserved regions amino acids compared to other Keratinase mojavisensis like proteins.

Phylogenetic tree

Alignment results were used to plot the phylogenetic tree. The amino acid sequences

for the proteins were called according to the NJ method in MEXA8 software, and a phylogenetic tree was plotted using an Out group (Figure 3).



Figure 3. The phylogenetic tree of Keratinase mojagensis. Green circle is Keratinase mojagensis and red circle is Subtilisin Homo sapiens.

Modeling Homology and Validation

The Keratinase mojagensis sequence was introduced in Blast-PDB and the 4GI3A structure was chosen as the base homology modeling. Since there is no Keratinase mojagensis crystallographic sequence, modeling was performed using Modeler software. Predictive model validation was performed by online software PROCHECK, PROSA and Geno3d. As shown in Figure 4A, the Z-Score point was set to -7.34. The Z-Score point is used to validate the modeled protein based on NMR or X-ray methods. The NMR method is used to crystallize proteins with less than 200 amino acids, which is more accurate than the X-ray method. In fact, if the Z-Score point in the PROSA graph is located on the (blue) NMR regions, the simulation accuracy is higher and the simulated model has the lowest error rate and the highest confidence. Also, in the graph plotted to determine the negative energy level of the protein if the amino acid sequence is found to be 10 and 40 times

lower than zero, the low energy level signifies greater protein stability and high value of simulated 3D data for the protein (Figure 4B). In the graph in Figure 4C, the red dots indicate the high-energy regions, and the blue dots indicate the low-energy areas in the protein.

Quality analysis of the predicted model after drawing Ramachandran graph showed that 90.4% of amino acids were in most favored regions, 6.2% in allowed regions, 3.4% in outlier regions. Overall, 98% of the amino acids are in their proper place. In the Ramachandran graph, amino acids are grouped according to the angles of ψ and ϕ (Figure 5). The final quality of the predicted model is acceptable. The VERIFY-3D program was used to find the compatibility of the predicted atomic model of the third predicted building of Keratinase mojagensis with the amino acid sequence of its first building. The compatibility score above zero in the VERIFY-3D graph is related to the degree of adaptability of the amino acid

sequence of Keratinase mojavensis in this model. The VERIFY-3D results for Keratinase mojavensis were higher than 2, which indicates that the predicted model is associated with high resemblance compared

to the 4GI3A structure (Figure 6). These results indicate that the predicted structure of Keratinase mojavensis has been successfully modeled and demonstrates the suitability for use in docking studies.

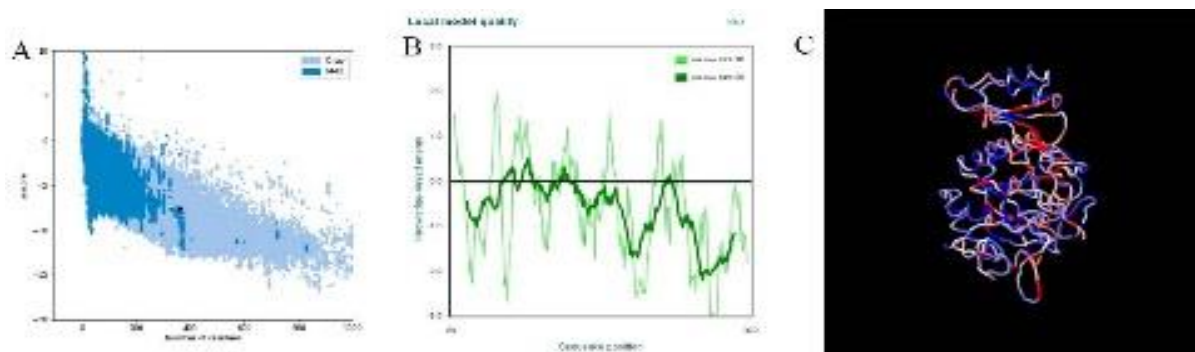


Figure 4. predicted homology modeling and structure validation of keratinase mojavensis. (a) the z-score graph for prosa. (b) local model quality. (c) the 3d structures of proteins energy.

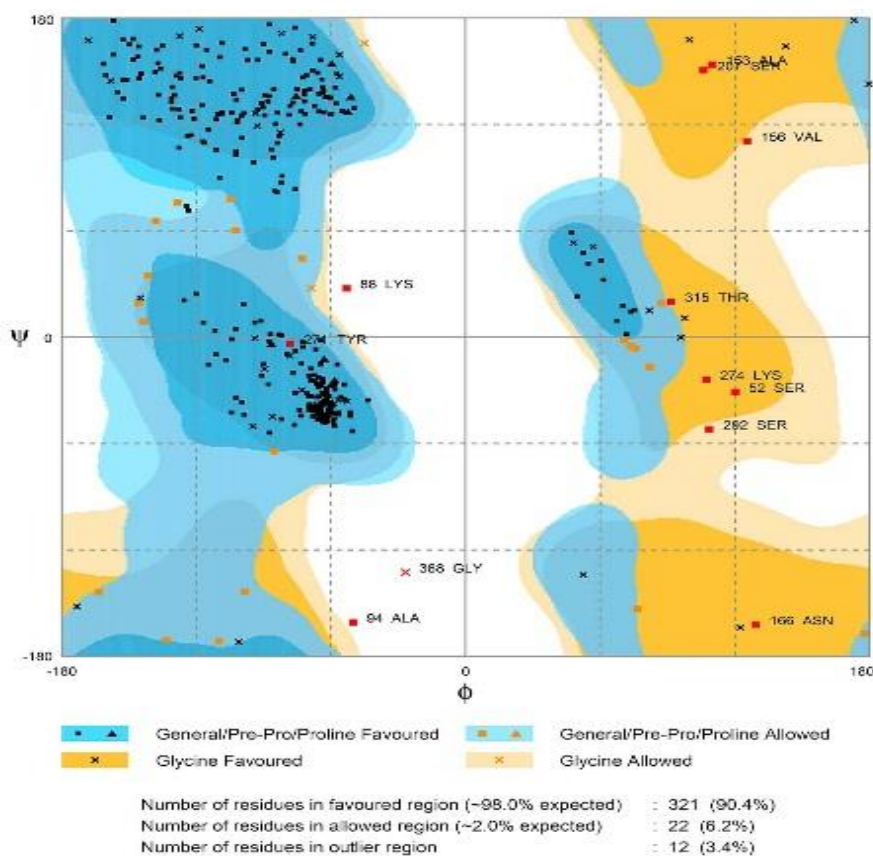


Figure 5. The Ramachandran graph. 99% of the amino acids in the predicted 3D structure are correctly angled in terms of the angles of ψ and ϕ .

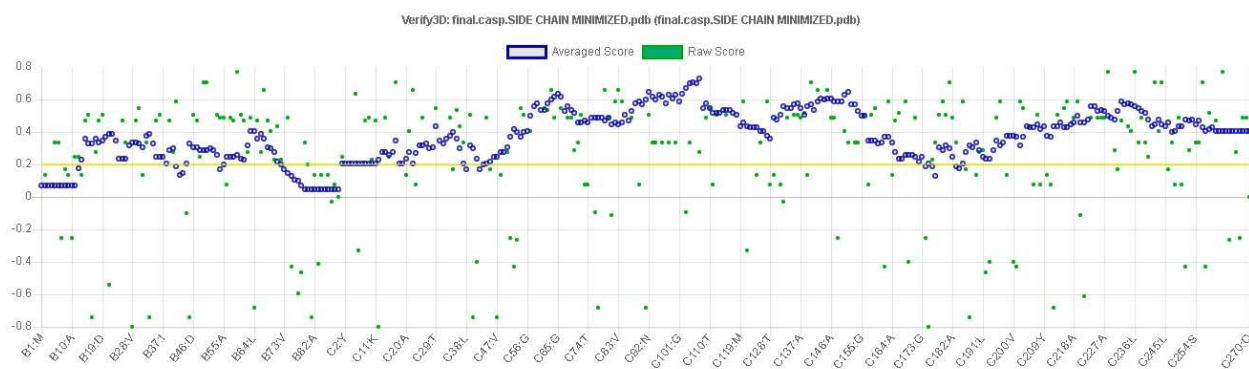


Figure 6. VERIFY-3D graph. Compatibility of the predicted atomic model of the third building of Keratinase mojavensis with the amino acid sequence of the first building of Keratinase mojavensis.

The PDB predicted model of Keratinase mojavensis by modeler and active site (Aspartate 137, Histidine 168, Asparagine 259 and Serine 325) prediction of Cofactor server is shown in Figure 7. Information on Molecular Function (MF) enzyme keratinase mojavensis is shown in the Predicted Gene Ontology (GO) Terms graph (Figure 8). As shown in Figure 8, the keratinase mojavensis is classified into a serine-type endopeptidase activity group.

As shown in Figure 9, superimposed using Chimera (RMSD=1.237) showed very high structural similarity between Keratinase mojavensis and 4GI3A.

Using the Chimera software, the best spatial positioning for optimal enzyme energy in the dock was achieved. This software calculates and estimates the total energy of the predicted structure of Keratinase mojavensis, making the protein ideally stable and energy-efficient (Figure 10).

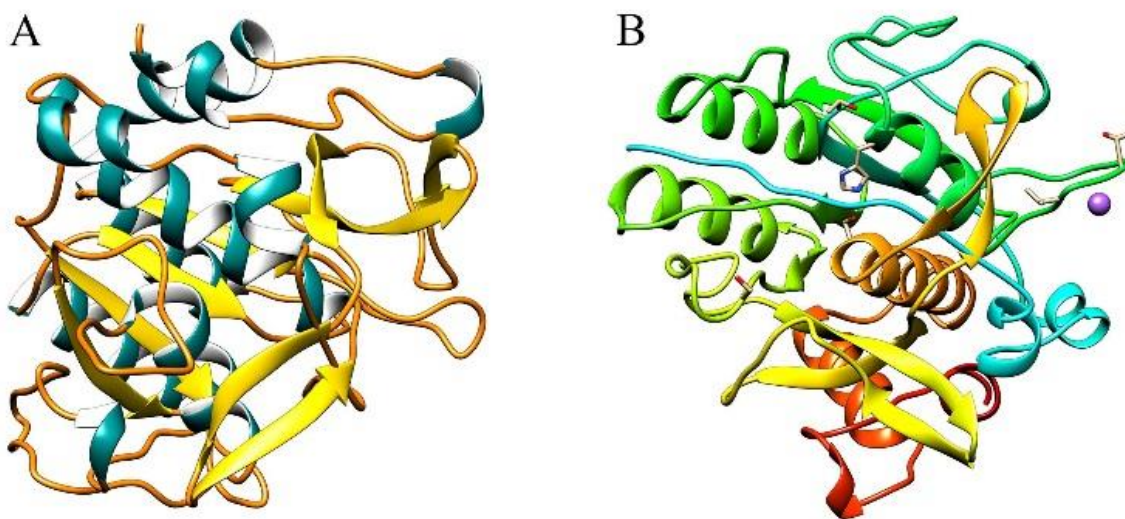


Figure 7. The predicted structure of Keratinase mojavensis. A) The predicted structure of Keratinase mojavensis by MODELER software. B) Prediction of Keratinase mojavensis active site by cofactor server.

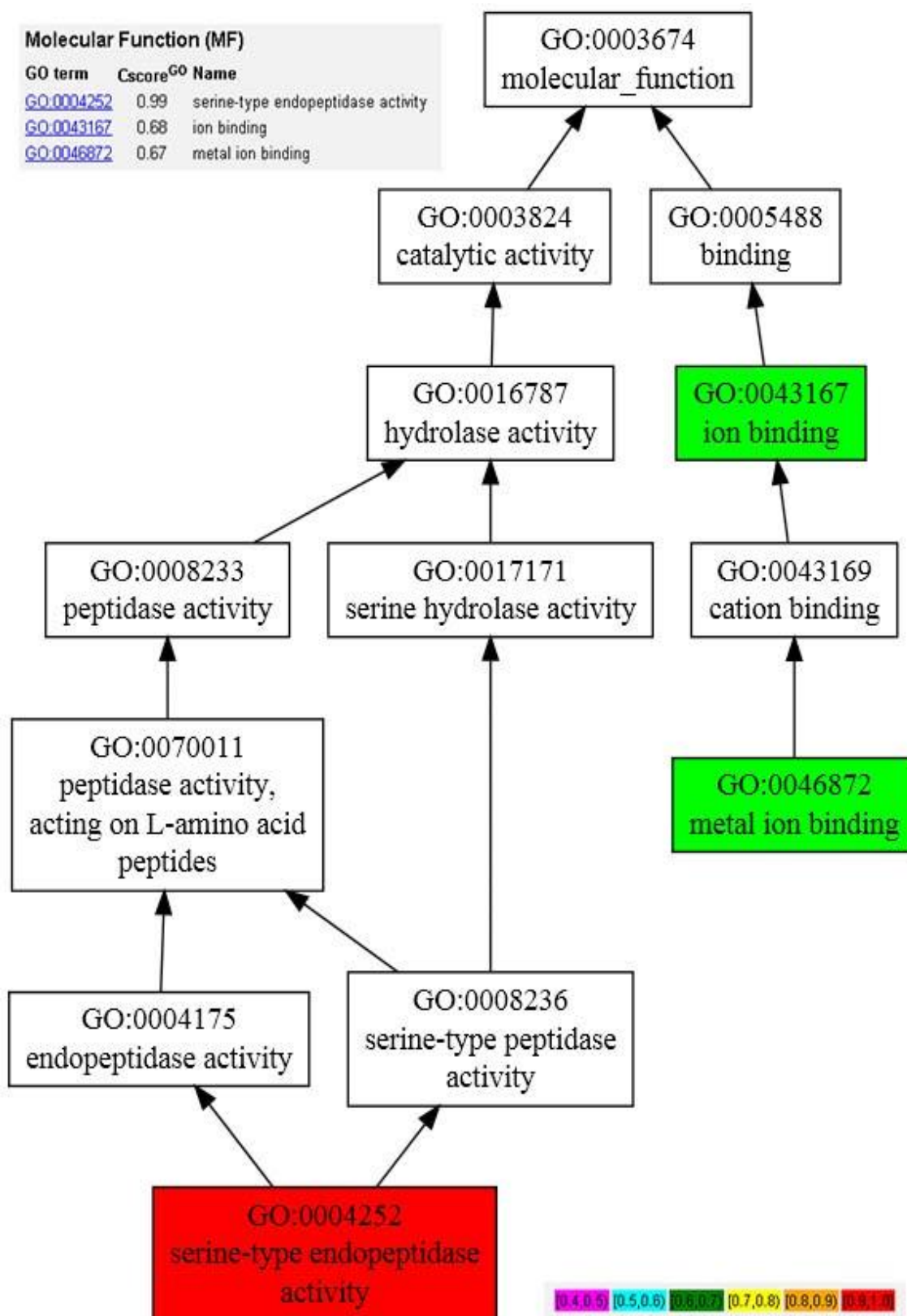


Figure 8. Cofactor's results of predicted gene ontology (GO) of keratinase mojavensis.

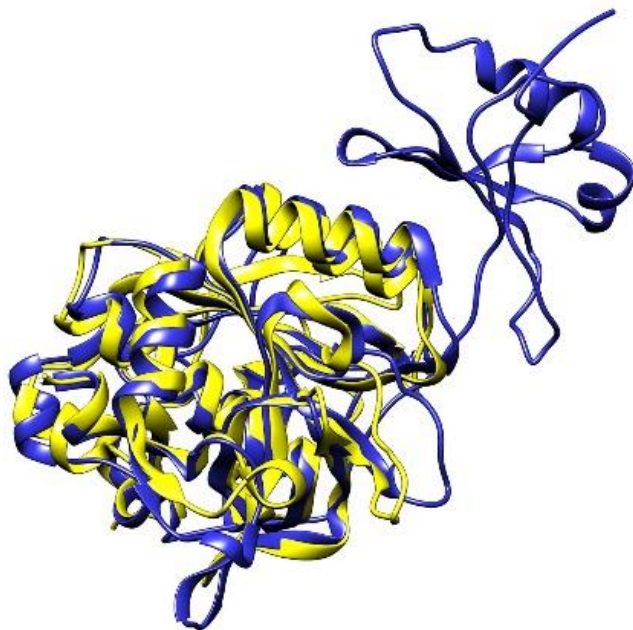


Figure 9. Superimpose the predicted 3D structure of Keratinase mojavensis and the 3D crystallographic structure of 4GI3A using Superimpose. Yellow: Predicted structure of Keratinase mojavensis, blue: 4GI3A 3D structure.

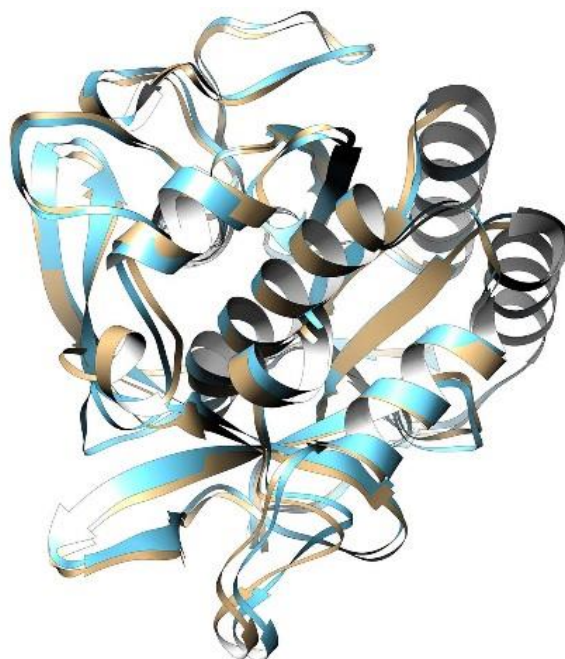


Figure 10. Superimpose form the 3D structure predicted by Keratinase mojavensis in Minimized mode with its 3D structure. Yellow: Predicted 3D structure of Keratinase mojavensis, Turquoise: Predicted 3D structure of Keratinase mojavensis in Minimized mode.

Docking studies

After the preparation of the ligands, the docking protein ligand was performed. As shown in Figure 11A&B, PMSF bonded 1 and 2 hydrogen bonds with serine amino acids 277 and glutamic 299, respectively. The 2D display of the interactions is shown in Figure 11C.

One of the powerful ways to detect the function of proteolytic enzymes in living organisms depends on the use specific inhibitors of this enzyme (23). One of the inhibitors that used in this study, is pepstatin. Pepstatin is an inhibitor of acid proteinase (24). As shown in Figure 12, this

inhibitor bonded a hydrogen bond with serine (277) and (295), leucine (230) and alanine (256) and possibly inhibits their catalytic activity.

The results of the leupeptin inhibitory effect are given in Figure 13. As shown in this figure, leupeptin binds hydrogen bonds with asparagine (265), serine (277 and 295), glutamic (299) and tyrosine (271) and exerts its inhibitory effect.

These results show one of the applications of using bioinformatics software and the role of protein-ligand docking technique in recognizing and understanding how the interaction of key amino acids on the catalytic site of keratinase enzyme.

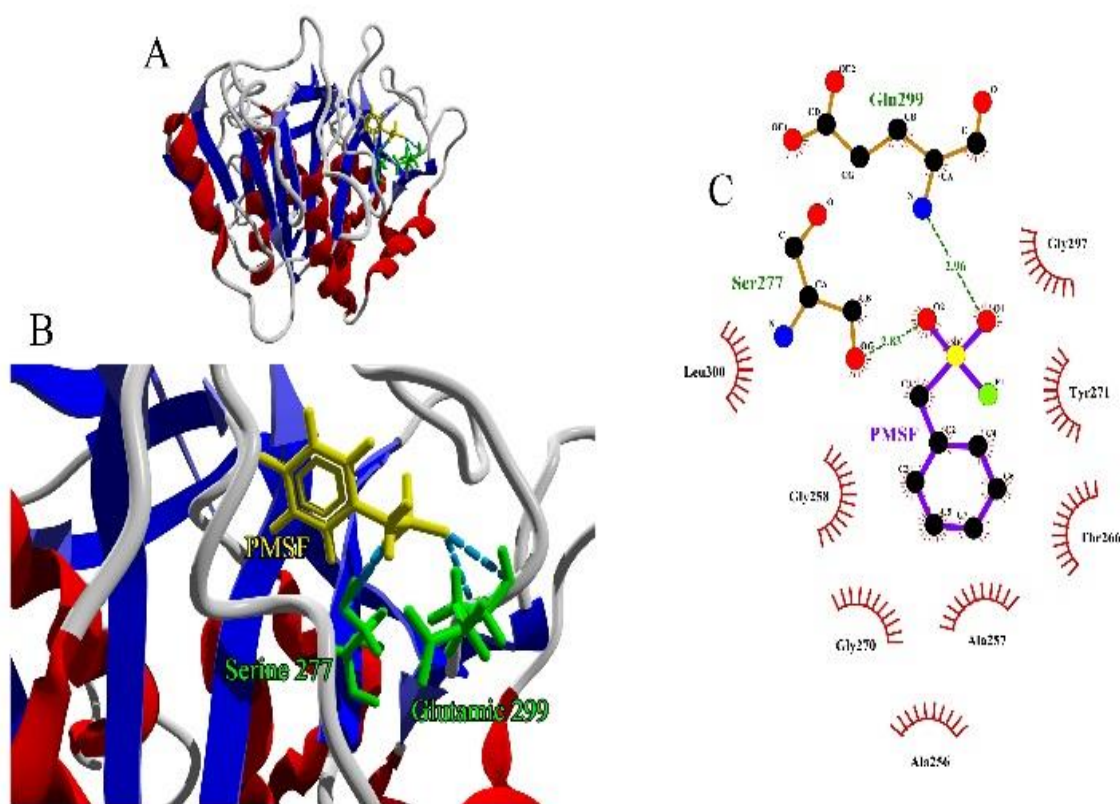


Fig 11. Docking of Mojavensis-PMSF Complex. (A) Mojavensis structure. (B) Mojavensis-PMSF complex. Mojavensis structure is shown as ribbon, α -Helix is red, β -sheet is blue, Coil is gray, ligand is yellow and H-Bond is dotted line blue. (C) 2D graph of the Mojavensis-PMSF complex. Ligand is purple, involved amino acids in complex are light brown, H-Bonds are dotted line green and side residues are red.

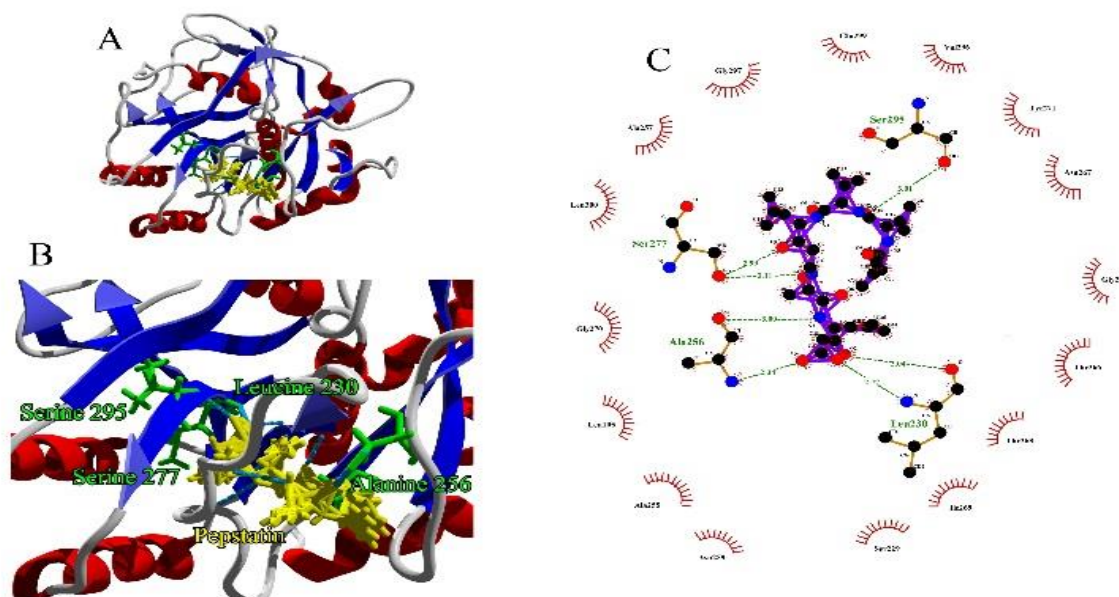


Fig 12. Docking of Mojavensis- Pepstatin Complex. (A) Mojavensis structure. (B) Mojavensis-Pepstatin complex. Mojavensis structure is shown as ribbon, α -Helix is red, β -sheet is blue, Coil is gray, ligand is yellow and H-Bond is dotted line blue. (C) 2D graph of the Mojavensis-Pepstatin complex. Ligand is purple, involved amino acids in complex are light brown, H-Bonds are dotted line green and side residues are red.

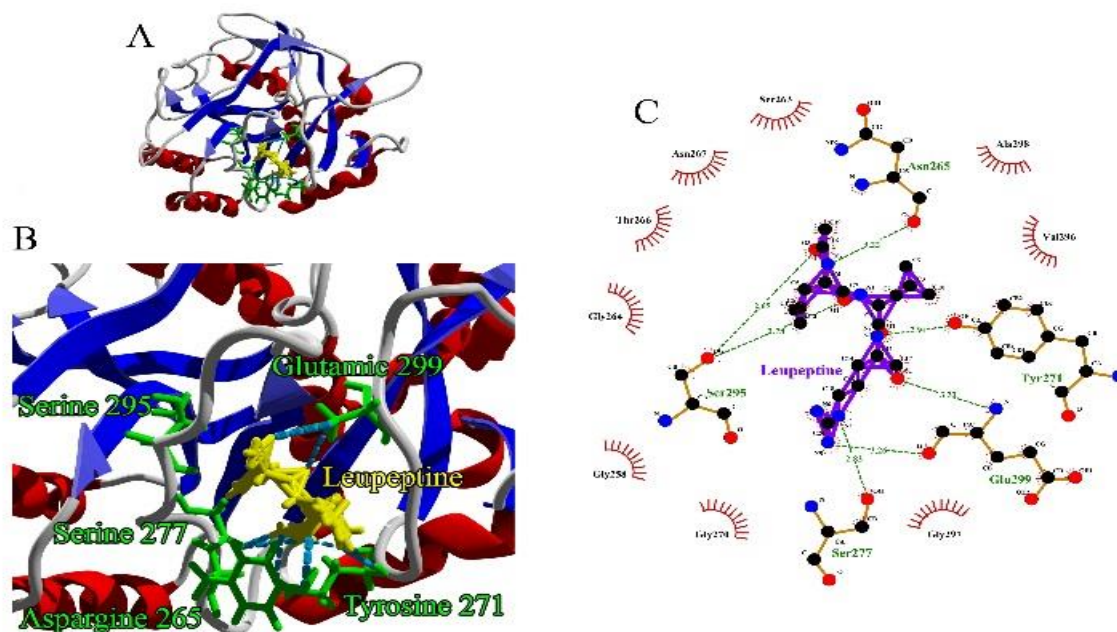


Fig 13. Docking of Mojavensis-Leupeptin Complex. (A) Mojavensis structure. (B) Mojavensis- Leupeptin complex. Mojavensis structure is shown as ribbon, α -Helix is red, β -sheet is blue, Coil is gray, ligand is yellow and H-Bond is dotted line blue. (C) 2D graph of the Mojavensis-Leupeptin complex. Ligand is purple, involved amino acids in complex are light brown, H-Bonds are dotted line green and side residues are red.

Discussion

Protein engineering is the process of artificially producing useful and valuable proteins. This is a new science that will allow experts in a variety of 2D and 3D protein structures to make various designs possible. In the biotechnology industry, protein engineering is specifically used to make enzymes. Since the determinant of the function of proteins is their 3D structure, computational modeling methods can be used to investigate this parameter (25). By using bioinformatics software, the effect of macromolecules on proteins (enzymes) can be predicted (26). Keratinase produced by microorganisms has many applications in animal feed, fertilizer production, the cleaning and leather industries, pharmaceuticals, and in fermentation technology. For example, keratinase from *B. licheniformis* PWD-1 and *Virio* sp. kr2 can be used to feed livestock (27). This enzyme can also reduce prion-induced infection with the presence of cleaners and temperature treatments. In the leather industry, keratinase from *B. subtilis* S14 has a significant ability to remove hair on the skin without causing the breakdown of collagen (28). One low-cost, cost-effective, accurate, and reliable way to identify protein structure is to predict the effects of inhibitors or enzymatic activity enhancer using bioinformatics methods such as molecular docking and molecular dynamics simulation (29). PMSF is a specific inhibitor of serine proteases. The functional mechanism of serine proteases is based on the activity of catalytic triad containing serine amino acids, histidine and aspartate. In this triad, the nucleophilic attack is carried out by the amino acid serine, while the amino acid histidine is the electron donor (30). In some types of peptidases, such as carboxypeptidases, glutamic amino acid contributes with serine and histidine instead of aspartate in catalytic activity (31). The PMSF's ability to bind

hydrogen to serine amino acids in this study was similar to that of Jayalakshmi et.al (32). Overall, the results of docking PMSF with keratinase *mojavensis* in this study confirmed the fact that PMSF has the ability to bind hydrogen bonds with amino acids of the active site from serine proteases such as creatinine and possibly inhibit them by blocking their catalytic site. Acid proteases rapidly attack peptide bonds that bind amino acids to hydrophobic side chains (23). Another inhibitor used in this study is pepstatin. Pepstatin is produced in culture media and fermentation tanks containing various carbon and nitrogen ratios (24). A normal culture medium for the production of pepstatin contains glucose, starch, peptone, meat extracts, NaCl and Mn, Fe and Zn minerals, and the maximum amount of pepstatin produced in these environments occurs after 5 to 6 days (24). In my study, pepstatin was able to hydrogen bonds with serine, leucine and alanine amino acids. Since 97% of the keratinase sequence is similar to alkaline proteases and is inhibited by serine protease inhibitors, it is referred to as a serine protease. But it has been sometimes found as a serine protease in combination with a cysteine protease and a metalloprotease (8). Leupeptin is a specific inhibitor of cysteine protease activity. Therefore, in this study, the effect of leupeptin inhibition was investigated on keratinase enzyme activity. Khara et al. (2020) examined the inhibitory effect of leupeptin on cathepsin B. In this study, it was found that leupeptin has a hydrogen bond with cathepsin B and has the ability to inhibit this cysteine protease. And as a result, according to bioinformatics studies, this inhibitor can be used to inhibit cathepsin B (33).

Conclusion

In this study the keratinase enzyme was predicted using bioinformatics software. As

the results showed, after 3D structure verification, it was found that the enzyme was modeled in the crystallographic regions. Predicting the active sites of the protein and determining the theoretical biochemical properties of the enzymes could aid in protein engineering studies to enhance

protein stability, study of proteins against inhibitors by docking, and molecular dynamics simulations.

Acknowledgments

The authors would like to thank all those who helped provide the project.

References

1. Choct M. Enzymes for the feed industry: past, present and future. *World's Poultry Sci J.* 2006;62(1):5-16. doi: 10.1079/WPS200480.
2. Kondamudi N, Strull J, Misra M, Mohapatra SK. A green process for producing biodiesel from feather meal. *J Agric Food Chem.* 2009;57(14):6163-6. doi: 10.1021/jf900140e.
3. Forgacs G, Lundin M, Taherzadeh MJ, Sarvari Horvath I. Pretreatment of chicken feather waste for improved biogas production. *Appl Biochem Biotechnol.* 2013;169(7):2016-28. doi: 10.1007/s12010-013-0116-3.
4. Fraser RD, Parry DA. Molecular packing in the feather keratin filament. *J Struct Biol.* 2008;162(1):1-13. doi: 10.1016/j.jsb.2008.01.011.
5. Khattak F, Pasha T, Hayat Z, Mahmud A. Enzymes in poultry nutrition. *J Anim Plant Sci.* 2006;16
6. Cai CG, Chen JS, Qi JJ, Yin Y, Zheng XD. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. *J Zhejiang Uni Sci.* 2008;9(9):713-20. doi: 10.1631/jzus. B0820128.
7. Mazotto AM, Coelho RR, Cedrola SM, de Lima MF, Couri S, Paraguai de Souza E, et al. Keratinase Production by Three *Bacillus* spp. Using Feather Meal and Whole Feather as Substrate in a Submerged Fermentation. *Enzyme Res* 2011; 2011:523780. doi: 10.4061/2011/523780.
8. Wang ZR, Qiao SY, Lu WQ, Li DF. Effects of enzyme supplementation on performance nutrient digestibility gastrointestinal morphology and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poultry Sci.* 2005;84(6):875-81. doi: 10.1093/ps/84.6.875.
9. Gupta R, Ramnani P. Microbial keratinases and their prospective applications: an overview. *Appl Microbiol Biotechnol.* 2006;70(1):21-33. doi: 10.1007/s00253-005-0239-8.
10. Zhexin X. Advances in homology protein structure modeling. *Curr Prote Pept Sci.* 2006;7(3):217-27. doi: 10.2174/138920306777452312.
11. Gasteiger E, Hoogland C, Gattiker A, Duvaud SE, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the expasy server. 1th ed. Totowa NJ Hum Publication. 2005; p. 571-607.
12. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mole Biol.* 2004;340(4):783-95. doi: 10.1016/j.jmb.2004.05.028.
13. Ceroni A, Passerini A, Vullo A, Frasconi P. DisulfInd a disulfideu bonding state and cysteine connectivity prediction server. *Nucle Acid Res.* 2006;34(4):177-81. doi: 10.1093/nar/gkl266.

14. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The phyre2 web portal for protein modeling prediction and analysis. *Nature Prot.* 2015;10(6):845-58. doi: 10.1038/nprot.2015.053.
15. Wass MN, Kelley LA, Sternberg MJ. 3Dligandsite predicting ligand binding sites using similar structures. *Nucle Acid Res.* 2010;38(2):469-73. doi: 10.1093/nar/gkq406.
16. Zhang C, Freddolino PL, Zhang Y. Cofactor improved protein function prediction by combining structure sequence and protein protein interaction information. *Nucle Acid Res.* 2017;45(1):291-9. doi: 10.1093/nar/gkx366.
17. Roy A, Yang J, Zhang Y. Cofactor an accurate comparative algorithm for structure-based protein function annotation. *Nucle Acid Res.* 2012;40(2):471-7. doi: 10.1093/nar/gks372.
18. Pontius J, Richelle J, Wodak SJ. Deviations from standard atomic volumes as a quality measure for protein crystal structures. *Journal of molecular biology.* 1996;264(1):121-36. doi: 10.1006/jmbi.1996.0628.
19. Wiederstein M, Sippl MJ. Prosa interactive web service for the recognition of errors in three dimensional structures of proteins. *Nucle Acid Res.* 2007;35(3):407-10. doi: 10.1093/nar/gkm290.
20. Sippl MJ. Recognition of errors in three-dimensional structures of proteins. *Prote Struc Func Bioinform.* 1993;17(4):355-62. doi: 10.1002/prot.340170404.
21. DePristo MA, de Bakker PIW, Johnson RJK, Blundell TL. Crystallographic refinement by knowledge-based exploration of complex energy landscapes. *Structure.* 2005;13(9):1311-9. doi: doi.10.1016/j.str.2005.06.008.
22. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF chimera visualization system for exploratory research and analysis. *J Comput Chem.* 2004;25(13):1605-12. doi: 10.1002/jcc.20084.
23. Barrett AJ, Dingle JT. The inhibition of tissue acid proteinases by pepstatin. *Biochem J.* 1972;127(2):439-41. doi: 10.1042/bj1270439.
24. Hamaoumezawa TA, Morishima H, Matsuzaki M, Hamada M, Takeuchi T. Pepstatin a new pepsin inhibitor produced by agtinomyetes. *Biochem Res J.* 2006;23(4):148-53. doi: doi.org/10.7164/antibiotics.23.259.
25. Engqvist MKM, Rabe KS. Applications of Protein engineering and directed evolution in plant research. *Plant Physiol.* 2019;179(3):907. doi: 10.1104/pp.18.01534.
26. Torchala M, Gerguri T, Chaleil RAG, Gordon P, Russell F, Keshani M, et al. Enhanced sampling of protein conformational states for dynamic cross-docking within the protein protein docking server Swarmdock. *Proteuu Struc Func Bioinform.* 2019;121(3):238-42. doi: 10.1002/prot.25851.
27. Odetallah NH, Wang JJ, Garlich JD, Shih JCH. Versazyme supplementation of broiler diets improve market growth performance. *Poult Sci.* 2005;84(6):858-64. doi:10.1093/ps/84.6.858.
28. Macedo AJ, Silva WOB, Gava R, Driemeier D, Henriques JAP, Termignoni C. Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. *Appl Environ Microbiol.* 2005;71(1):594. doi: 10.1128/AEM.71.1.594-596.2005.
29. Dagliyan O, Proctor A, Dauria M, Ding F, Dokholyan V. Structural and dynamic determinants of protein peptide recognition. *Structure.*

- 2011;19(12):1837-45. doi: 10.1016/j.str.2011.09.014.
30. Di Cera E. Serine proteases. IUBMB Life. 2009;61(5):510-5. doi: 10.1002/iub.186.
31. Blow DM, Birktoft JJ, Hartley BS. Role of a buried acid group in the mechanism of action of chymotrypsin. Nature. 1969;221(5178):337-40. doi: 10.1038/221337a0.
32. Jayalakshmi TPK, Rameshkumar G, Sivamani P. Docking conformation of *Bacillus licheniformis* keratinase enzyme and PMSF ligand. J Prsol Info. 2011;55(972):436-9.
33. Khara A, Jahangirian E, Tarrahimofrad H. The homology modeling and docking investigation of human cathepsin B. Int J Med Toxicol Forens Med. 2020;10(1):26687. doi: 10.32598/ijmtfm.v10i1.26687.