# Bplant: Solving Traditional Production Issues of Bromelain by Directed Evolution

**Jiaying Wen, Jingxian Cai, Zhenxiang Guo, Huiwen Cheng, Jingyi Ye**

## Abstract

Bromelain is a plant protease that can transform protein in feed into polypeptides and small peptides that are easy for animals to digest and absorb. Its application in the field of fodder processing brings a variety of benefits. Even though bromelain has great application potential in feed industry, the traditional production process has issues of inefficiency, unstable source and low enzyme activity. So we hope to improve the enzyme activity and stability by directed evolution. There are four main tools we used: Hotspot Wizard (for screening sites that can be mutated) , Pymol (to display protein structure) , Relax (to calculate energy to screen mutants) and Hdock (protein-molecule docking). Finally, we choose a double mutation model of glycine 16 and leucine 67. We've tested our results with Molecular Dynamics Simulations and wet experiments.

**Keywords**: bromelain, directed evolution, activity, stability, feed

## Introduction

Bromelain is a group of thiol hydrolytic proteases extracted from the tropical plant pineapple and mainly exists in the fruit, bud, leaf and stem of pineapple, with a molecular weight of 33000. It belongs to the papain family of cysteine proteases, and the enzymatic activity is dependent on the thiol group of a cysteine residue within its active site. Bromelain has a variety of properties, including anti-cancer activity, anti-inflammatory effect, antimicrobial effect, antibiotic potentiation, skin protection, postsurgery recovery and so on[1-3]. Therefore, it has a wide range of applications in the medical[3] and food fields[4]. We choose fodder processing as the application scenario of the study. Bromelain is a plant protease, which can convert protein in feed into peptides and small peptides easily absorbed by animals, improve the conversion

rate of feed, so as to reduce the pollution of breeding industry to the environment[5,6]. It also has a certain therapeutic effect on diarrhea caused by pathogenic bacteria, avoiding the negative effects of antibiotics[5], improving the growth performance of animals[7,8], and resisting the damage of parasites to animals[5].

However, the traditional production technology has the problems of high cost, low enzyme activity and low yield because it takes pineapple as raw material[5]. At the same time, the processing process of this traditional technology will bring some environmental resources problems[9]. Therefore, under the background of shortage of protein feed resources and low efficiency of protein utilization of livestock and poultry in China, an alternative method to produce highly active bromelain is very desirable. We hope to use the engineered bacteria to heterogeneously express bromelain, so as to ameliorate the traditional production methods. Bromelain has been recombinant expressed in Escherichia coli in the current literature[10,11], so we plan to use *E. coli* to express it.

In order to get better bromelain with higher enzyme activity, we carried out directional evolution on the selected target fragments. The screening tool for point mutations was Hotspot Wizard, the 3D structure of the protease was demonstrated by Pymol, and the protease stability after mutation was calculated by RELAX section in R2, an online platform. After Hotspot Wizard is used to screen out the possible mutation sites, pymol is used to mutate them to the amino acids suggested by Hotspot Wizard, and then the mutation with the lowest energy is selected as the target variant. Second mutation is carried out on this variant to obtain our optimal enzyme mutant. Next, we used Molecular Dynamics Simulations to verify the stability of bromelain reaction with BAEE.

## Methods and Materials

### Selection of target segments

The AlphaFold2 platform was used to search for bromelain. Two different stem bromelain sequences were found, one with 212 amino acids and the other with 291 amino acids. So we must choose which one to use as the target sequence, and this selection process requires comparing the two sequences. The global protein sequence alignments of these two sequences were performed by Clustal W. The 291-amino acid stem bromelain sequence had extra 122 amino acids at the beginning and the 212-amino acid stem bromelain sequence had extra amino acids at the end. However, there was a length of constant amino acids in the middle of both sequences that are highly similar. Clustal W showed that the amino acids in this region are extremely conserved. At the same time, 291 and 212 structure comparison analysis was conducted. The RMSD value was 0.42. It could be seen that they were highly similar in structure, which also indicated that both of them were stem bromelain. Their structure was shown below（Figure 1）.

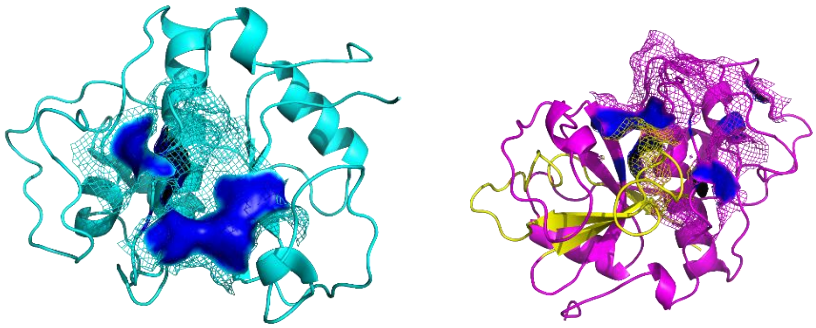

Figure 1

Finally, we chose the stem bromelain sequence containing 212 amino acids as target segment as it was reviewed and thus, more reliable.

## Construction of point mutation model

In order to enhance bromelain enzyme activity, we carried out directional evolution on the selected target fragments. The screening tool for point mutations was Hotspot Wizard, the 3D structure of the protease was demonstrated by Pymol 2.4, and the protease stability after mutation was calculated by RELAX section in R2, an online platform.

After Hotspot Wizard is used to screen out the possible mutation sites, Pymol 2.4 is used to mutate them to the amino acids suggested by Hotspot Wizard, and then the mutation with the lowest energy is selected as the target variant. Second mutation is carried out on this variant to obtain our optimal enzyme mutant.

## Synthesis of the target fragment

Our target segment was synthesized by our company (Engines, Nanjing). The company also performed two point mutations (serine at position 16 and leucine at position 67) and codon optimization for Bacillus coli.

## Molecular Docking Analysis

The simulation of molecular docking was performed on the Hdock protein-protein docking server. The docking results were visualized in the Hdock to confirm the binding position of bromelain and the BAEE.

## Molecular Dynamics (MD) Simulation

The dynamic behavior of Stem Bromelain (212aa) and BAEE complexes were studied by MD simulation performed on Gromacs.2020-fosscuda-2019b using the CHARMM36 all atom force field. The solvation of the system was done by using the TIP3P water model with a margin of 1Å. The system was neutralized by the addition of ions(NA and CL). Thenceforward, a consecutive minimization step was performed before the long MD simulation. 50000 steps were submitted and atom coordinates for the whole system were restrained to their initial coordinates with a force constant of 1000 KJ/mol Å−2. Before starting the molecular simulation, the temperature and pressure balance of the whole system was necessary. (Each balancing process took 50,000 steps.) The pdb2pqr module of Gromacs was used for protein-protein complex preparation and the gmx programs was used for counter ion addition, solvation, and preparation of topology files. Gromacs.2020-fosscuda-2019b on Beikun Cloud

supercomputing platform was used for MD simulation production and trajectory processing.

## Expression Vector

Sequence corresponding to the bromelain expression is synthesized by the methods mentioned above and cloned as BamHI- XhoI inserts in the pET-32a expression vector. pET-32a (Tsingke) is a commonly used T7 promoter based vector which can propagate in Escherichia coli and express the recombinant protein after appropriate .The desired polypeptide can be expressed as a fusion protein with 6xHis tag at the C-terminus for simplified purification.

## Inducible expression of pineapple protease

To enhance the expression of the heterologous protein, the strain used in this experiment was the rne131 mutant *E. coli* BL21 Star (DE3), from Tsingke Biotechnology Co. All strains used in this study were cultured in Luria-Bertani (LB) medium containing Ampicillin at 37° C and 220 rpm. After transformation experiments using the above strains, a colony of the mutant strain was selected from a Petri dish and inoculated into 3mL of LB liquid medium containing Ampicillin ( 37° C) in a 15mL centrifuge tube and incubated overnight at 37° C with 220rpm shaking. The temperature and speed were kept constant and iptg was added to the system to induce 3-4h, after which the bacteria were lysed for protein isolation and purification.

## Protein Purification

Since our bromelain product is a recombinant product, in order to obtain a relatively pure protease that can be characterized in biochemical analysis, we should eliminate all other miscellaneous proteins as much as possible. Although many of the purification methods are theoretically feasible, the properties of enzymes and other proteins might have not been distinguished. Therefore, the best way is to highlight the "specificity" of the recombinant protein. A more classical and reliable method is to

introduce a "tag" on the recombinant protein and take the tag as the focus of separation and purification, so that targeted separation can be carried out.

### A His-tag modification method

The residue of histidine (His) has an imidazole group which can form coordination bonds with transition metal ions such as Ni2+, Co2+ and can selectively bind to metal ions. These metal ions can be immobilized on the chromatography medium with chelating ligands. Therefore, the protein with his tag can selectively bind to the medium when passing through the chromatography medium equipped with metal ions.

While constructing the protease, we can add a DNA sequence corresponding to 6 consecutive histidine residues at the N-terminal or C-terminal of the protein, thus obtaining the original purification tag. [12] [13]

### Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC), however, is the mainstream method for protein purification, especially for the purification of a His-tag modified protein. The tetracoordinated NTA azatriacetic acid can form a very stable structure with high protein loading and low metal ion shedding, which makes Ni-NTA filler a good choice for pure his-tag modified protein. [14]

In the last step of protein purification, we apply to a highly concentrated imidazole solution to elute the protease with His-tag. Since the protein is not suitable for long-term storage in this system, we need to replace the high concentration imidazole with a buffer suitable for long-term preservation of proteins. Therefore, we still need to exchange the original imidazole solution with the new buffer by means of gel chromatography.

With the help of that, we can now elute and exchange the protein product stock

solution with the protein buffer we want. The original imidazole molecule and metal salt ions will be adsorbed by polyacrylamide resin.

### Purification Procedure

Binding of the poly histidine-tagged proteins can be performed using either a column or a batch procedure. Cell lysis could be done in buffered solution adjusted to pH 7.5-pH 8.0. We choose a column procedure in pursuit of more controllability. When the column procedure is utilized, the resin is packed into a column and the cell lysate is slowly loaded (we approximately add 3 to 4 column volumes per hour) onto the column. However, the use of the minimum amount of resin needed to bind the tagged protein is recommended. [15]

The tagged protein usually has a higher binding affinity than other proteins that bind nonspecifically to the resin. Thus, when the minimum amount of resin is used, the tagged protein will fill most of the available binding sites, reducing the number of nonspecific proteins that bind. Sodium chloride (we use a 250mM concentration) and low levels of imidazole (10mM to 20mM at most) are also included in the binding buffer to reduce the number of proteins that bind nonspecifically to the resin. Following binding of the tagged protein, the column can be washed to remove nonspecific proteins that bind weakly to the column. If desired, the inclusion of imidazole (20mM is used) in the wash buffer will increase the stringency of the wash and elute nonspecifically bound proteins more effectively.

Lowering the pH protonates the imidazole nitrogen atom of the histidine residue and disrupts the coordination bond between the histidine and the transition metal. [16] The histidine analog imidazole can be used to competitively elute the bound poly histidine residues so a high concentration of imidazole (in spite of a gradient we use a 250mM concentration) is applied for elution.

### Protein analysis and characterization

The purpose of SDS-PAGE is to effectively monitor the efficiency of protein concentration in the experiment, and to judge the risks and operational errors of some steps and improve the operations and steps that need to be paid attention to in the subsequent experiments.

# Results

## Search for mutable sites to obtain superior variants

Table 1 and 2 show the point mutation sites provided by Hotspot Wizard. In the search for point mutations, reference was made to the original analysis of residue 291. All conserved residues in enzymes are composed of polar, nonpolar, acidic and basic groups. Most of the conserved residues, such as Tyr, Asn, Gly, Thr, Gln and Cys, belong to polar groups, followed by non-polar groups (Phe, Trp, Ala, Val and Pro), acidic groups (Glu and Asp) and basic groups (Arg and His). So we evaluated some functional residues as well as other residues.

Table 1 Mutable structural residues

| Site | Original residues | Mutational residues |
|---|---|---|
| 18 | Lys | Arg or Gln |
| 20 | Gln | Arg |
| 51 | Gln | Ala |
| 159 | Val | Phe or Leu |

Table 2 Mutable nonstructural resideus

| Site | Original residues | Mutational residues |
|---|---|---|
| 16 | Ser | Gly or Asp |
| 161 | Thr | Phe or Leu |
| 172 | Tyr | Ile |

Rosseta's Relax module was used to score these point mutation models. The initial model energy was calculated as -607.691. The energy of No. 18 Arg mutation was -332.407 and the structure was disorganized（Figure 2）, so the mutation scheme has been abandoned. The possible reason was that site 18 was mutated into Arg, and the surrounding area was positively charged, while Arg was negatively charged in the past, and the energy was too high, so the structure appeared to fall apart.

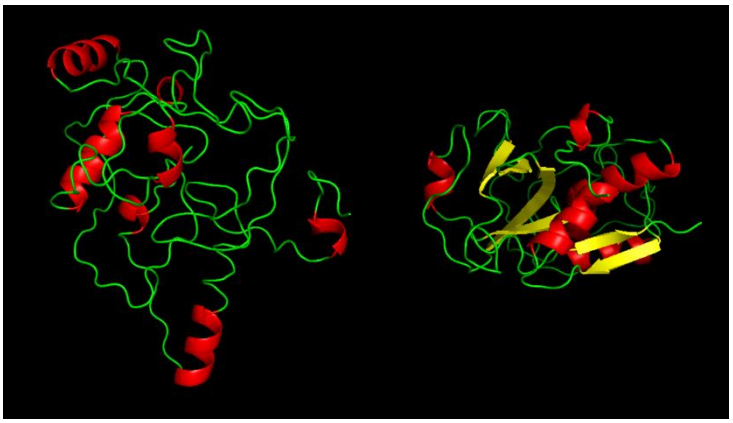

Figure 2

The energy of the Gln mutation 18 was -605.077. Although its structure did not fall apart, its energy increased and thus it was abandoned. The structure of Arg 20 was disintegrated after mutation, so it was abandoned too. (Figure 3)

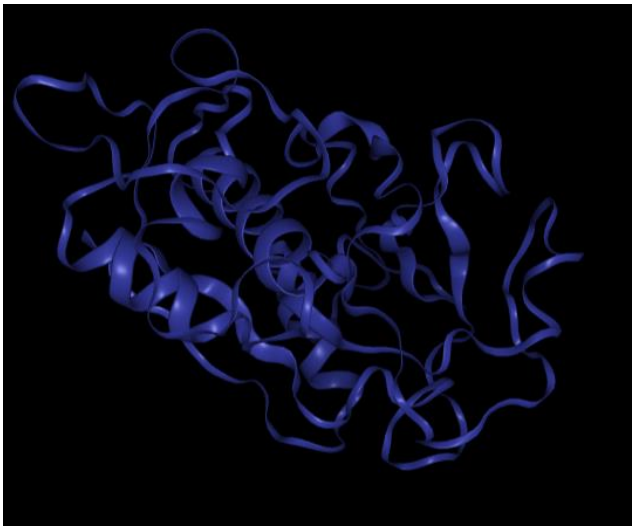

Figure 3

However, the energy of Gly 16 was -610.561 after mutation. Meanwhile, compared with the structure before mutation, its RMSD value was 0.683, so it was selected for further mutation.

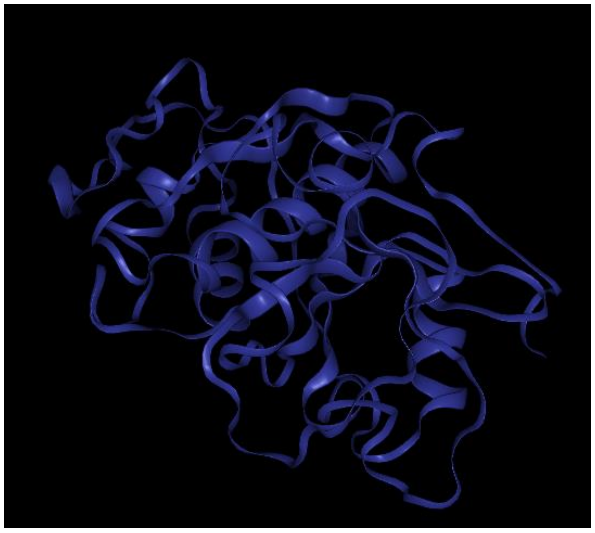

Figure 4

Finally, we screened the model with Leu mutation at site 67, and its energy was -614.826. Compared with the original model, the RMSD value was 0.97. Its reduced energy and relatively stable structure suggested that it might be a better variant of bromelain, as the experiments would prove.

## Exploration of upper relationship between residues of stem bromelain

The definition of upper relationship means that some single mutations of A are unfavorable, but the addition of B mutation will have better performance than single B mutation, which means that B has upper relationship with A. After calculating by Relax, we found that the Gly single mutation at site 16 had an upper relationship with the Leu single mutation at site 67: when Leu single mutation was detected, the structure was disordered, RM value was 1.4, and energy was -348. But with the addition of Gly, the structure stabilizes and the energy drops to -614.82. At the same time, the single mutation of Gly at site 16 has an upper relationship with the single mutation of Val at site 64: the energy of single mutation of Val is -599, while the energy of mutation of both Gly and Val decreases to -608.

## Substrate docking of stem bromelain and its variant

After literature review and network search, BAEE was finally determined as the

reaction substrate of bromelain, and its docking and energy calculation were performed with bromelain and mutated variants. The calculations were done online by Hdock.

Finally, the binding energy of bromelain in wild-type was -125.09, while that in mutant was -126.26, which decreased. This also proves that the stem bromelain variant we developed is more easily bound to the substrate and its reactivity is enhanced（Figure 5, 6, 7 and 8）

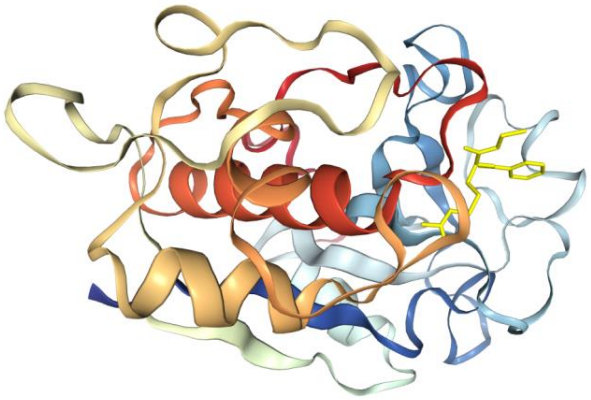

Figure 5

**Summary of the Top 10 Models**

| Rank | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Docking Score** | -125.09 | -122.33 | -121.09 | -120.68 | -118.88 | -115.88 | -115.26 | -114.81 | -114.01 | -113.65 |
| **Confidence Score** | 0.3780 | 0.3651 | 0.3593 | 0.3575 | 0.3492 | 0.3357 | 0.3330 | 0.3310 | 0.3274 | 0.3259 |
| **Ligand rmsd (Å)** | 15.10 | 18.46 | 17.58 | 12.64 | 17.11 | 25.16 | 13.18 | 16.27 | 12.77 | 20.94 |
| **Interface residues** | model_1 | model_2 | model_3 | model_4 | model_5 | model_6 | model_7 | model_8 | model_9 | model_10 |

Figure 6

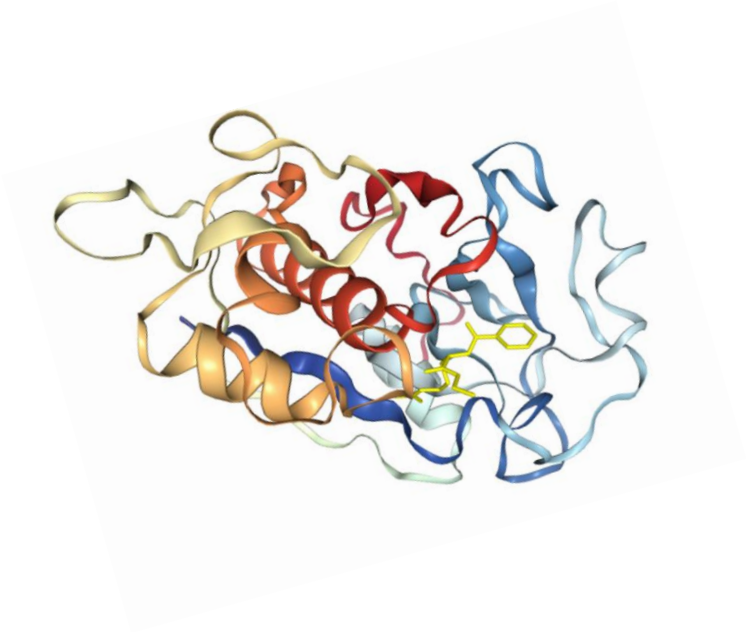

Figure 7

**Summary of the Top 10 Models**

| Rank | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Docking Score | -126.26 | -122.02 | -120.19 | -119.96 | -119.70 | -118.43 | -115.58 | -114.32 | -113.19 | -111.70 |
| Ligand rmsd (Å) | 11.30 | 15.32 | 19.83 | 14.89 | 14.02 | 14.53 | 19.54 | 12.31 | 14.65 | 15.05 |
| Interface residues | model_1 | model_2 | model_3 | model_4 | model_5 | model_6 | model_7 | model_8 | model_9 | model_10 |

Figure 8

## Molecular Dynamics Simulation Analysis

A molecular dynamics simulation was conducted to analyze the binding stability of Stem Bromelain(212aa) and BAEE complexes, where multiple descriptors were analyzed to understand the flexible and stable nature of the complexes. The system has been balanced in advance (the result of temperature and pressure balance is shown in below(Figure 9,10).

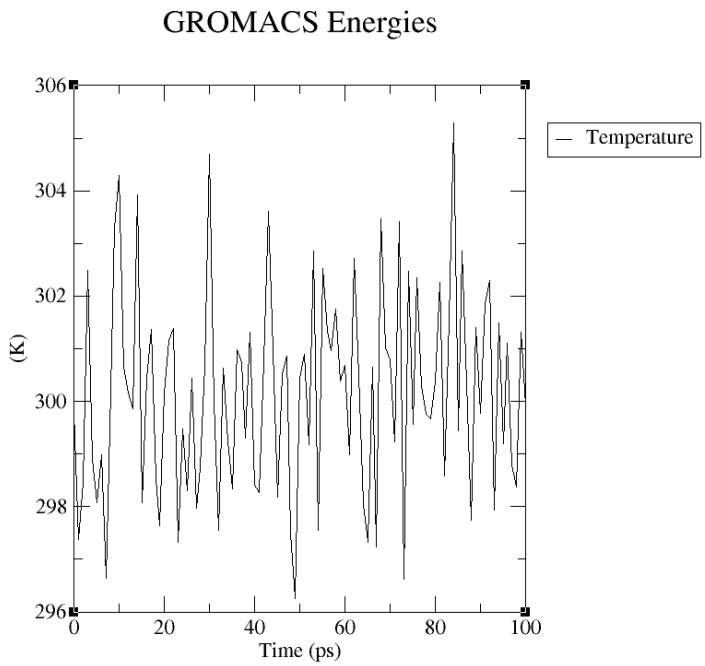


Figure 9: the result of temperature balance

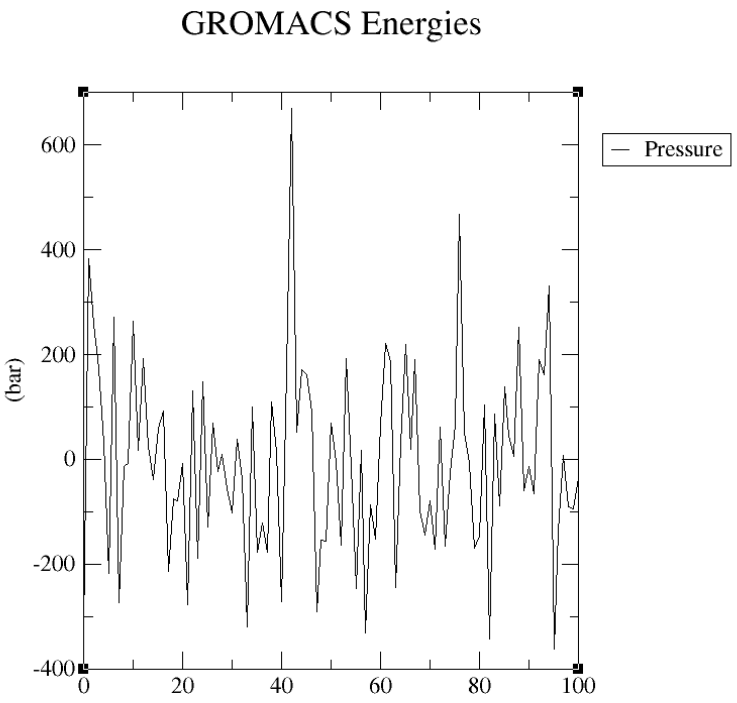


Figure 10: the result of pressure balance

After the whole system was balanced, the molecular simulation started to run, which took 10h (1ns) in total.

RMSD and RMSF analysis were performed on the simulated results, and the results were shown in the figure below. It can be seen that Stem bromelain(212) has good

reactivity with BAEE in neutral environment. The RMSD value was less than 0.4, and the RMSD value structure did not change much before and after simulation, indicating that the reaction between the complex was very stable. At the same time, the RMSF value changed greatly, which reflected that the atomic motion of Stem bromelain (212) was relatively free when it reacted with BAEE.

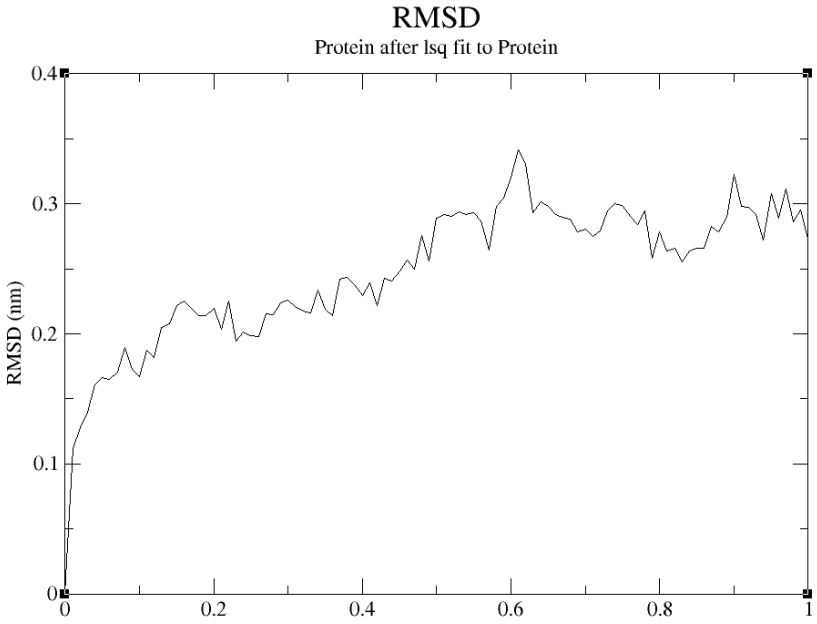


Figure 11: the RMSD of Stem Bromelain(212) and BAEE complex

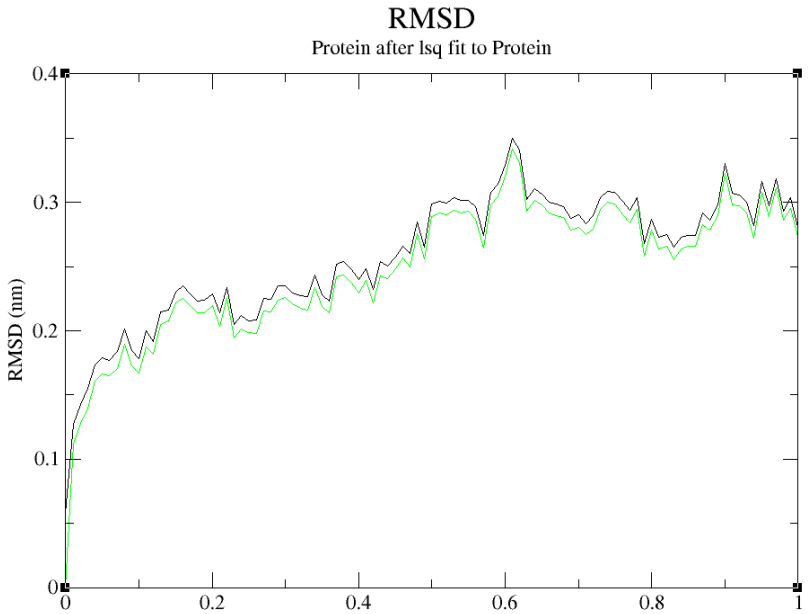


Figure 12: the comparing RMSD of Stem Bromelain(212) and BAEE complex

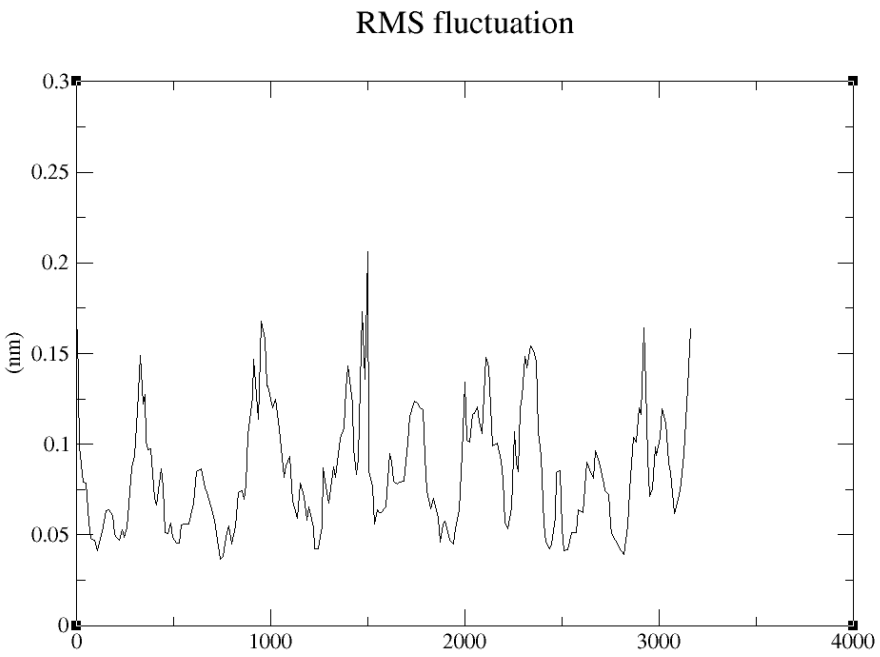


Figure 13: the RMSF of Stem Bromelain(212) and BAEE complex

As for the mutant, since it can also dock with BAEE and its binding energy is better than that of Stem Bromelain (212), we predict that its RMSD map fluctuation should be smaller, and the data will be made up in the later molecular simulation.

## Expression of Bromelain

Bromelain wild type, bromelain variant 1 and bromelain variant 2 were synthesised at 37°C, pH=7 and induced by iptg (50mg/mL, Sangon Biotech). After 3-4 hours all sluices containing the three proteins were collected and sonicated to lyse the bacteria. Correctly transformed and induced E. coli expressed bromelain wild type, bromelain variant 1 and bromelain variant 2 (Figure 14). We can see the bromelain (variant) bands around 25-35 kDa. By isolating and purifying pineapple protease variant 1 and variant 2 by Ni columns, we obtained a protein solution containing all three of these proteins.

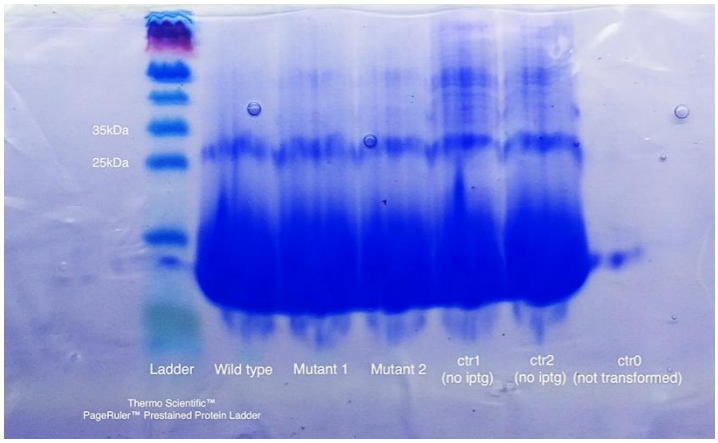


Figure 14: Electrophoresis of proteins before and after transformation

## Discussion

Stem Bromelain is an enzyme widely used in industry, food processing and other fields. However, due to the low activity of the enzyme in industrial production, it is very important to develop a variant with better activity and stability. We adopted rational directed evolutionary design, started with site-directed mutagenesis, and finally obtained a variant with excellent performance. In the future, we will use molecular simulation and other means to further investigate the properties of this mutant, so as to clarify the advantages of this mutant over the wild-type. At the same time, we will also develop a variety of mutants and compare their properties, in order to look forward to the research and development of more excellent mutants, which will also have a certain enlightenment for the future work of the team.

After isolation and purification of the three proteins, wild-type pineapple protease, pineapple protease variant 1 and pineapple protease variant 2, we will carry out the corresponding physicochemical properties, enzyme activity assays and characterisation experiments. We will rigorously determine the optimum temperature, pH and thermal stability of the enzymes and characterise them in specific application scenarios (e.g. feed, soybean meal characterisation) to verify the evolutionary goals of thermal stability, high enzyme activity and high quality. We'll carry out the corresponding modeling in the future.

We will also use safer, more application-friendly chassis microorganisms for recombinant expression. For example, we have specifically designed the use of the probiotic Bacillus subtilis as the chassis microorganism in feed applications, followed by the corresponding single-factor, orthogonal and characterisation experiments, and the use of a directed evolution of bromelain in feed applications to verify the validity of our work.

## References


[1] Shin H, Kim HT, Choi MJ, Ko EY. Effects of Bromelain and Double Emulsion on the Physicochemical Properties of Pork Loin. Food Sci Anim Resour. 2019 Dec;39(6):888-902. doi: 10.5851/kosfa.2019.e70. Epub 2019 Dec 31. PMID: 31950106; PMCID: PMC6949528.

[2] Hikisz P, Bernasinska-Slomczewska J. Beneficial Properties of Bromelain. Nutrients. 2021 Nov 29;13(12):4313. doi: 10.3390/nu13124313. PMID: 34959865; PMCID: PMC8709142.

[3] Shin H, Kim HT, Choi MJ, Ko EY. Effects of Bromelain and Double Emulsion on the Physicochemical Properties of Pork Loin. Food Sci Anim Resour. 2019 Dec;39(6):888-902. doi: 10.5851/kosfa.2019.e70. Epub 2019 Dec 31. PMID: 31950106; PMCID: PMC6949528.

[4] Chakraborty AJ, Mitra S, Tallei TE, Tareq AM, Nainu F, Cicia D, Dhama K, Emran TB, Simal-Gandara J, Capasso R. Bromelain a Potential Bioactive Compound: A Comprehensive Overview from a Pharmacological Perspective. Life (Basel). 2021 Apr 6;11(4):317. doi: 10.3390/life11040317. PMID: 33917319; PMCID: PMC8067380.

[5] Wang XQ, Liang SJ, Zhou JY, Gao CQ, Yan HC. Application prospect and overview of bromelain on feed [J].Feed Industry,2016,37(04):1-4.DOI:10.13302/j.cnki.fi.2016.04.001.

[6] Dutta S, Bhattacharyya D. Enzymatic, antimicrobial and toxicity studies of the aqueous extract of Ananas comosus (pineapple) crown leaf. J Ethnopharmacol. 2013 Nov 25;150(2):451-7. doi: 10.1016/j.jep.2013.08.024. Epub 2013 Sep 26. PMID: 24076462.

[7] Choi WM, Lam CL, Mo WY, Wong MH. Upgrading food wastes by means of bromelain and papain to enhance growth and immunity of grass carp (Ctenopharyngodon idella). Environ Sci Pollut Res Int. 2016 Apr;23(8):7186-94. doi: 10.1007/s11356-015-4863-2. Epub 2015 Jun 20. PMID: 26092357.

[8] Contreras A, Paape MJ, Miller RH, Corrales JC, Luengo C, Sánchez A. Effect of bromelain on milk yield, milk composition and mammary health in dairy goats. Trop Anim Health Prod. 2009 Apr;41(4):493-8. doi: 10.1007/s11250-008-9213-7. Epub 2008 Jul 27. PMID: 18661260.

[9] Fouz N, Amid A, Hashim YZ. Cytokinetic study of MCF-7 cells treated with commercial and recombinant bromelain. Asian Pac J Cancer Prev. 2014 Jan;14(11):6709-14. doi: 10.7314/apjcp.2013.14.11.6709. PMID: 24377593.

[10] Azura Amid, Nurul Azira Ismail, Faridah Yusof, Hamzah Mohd Salleh. Expression, purification, and characterization of a recombinant stem bromelain from Ananas comosus.Process Biochemistry, Volume 46, Issue 12, 2011, Pages 2232-2239, ISSN 1359-5113. doi:10.1016.

[11] George S, Bhasker S, Madhav H, Nair A, Chinnamma M. Functional characterization of recombinant bromelain of Ananas comosus expressed in a prokaryotic system. Mol Biotechnol. 2014 Feb;56(2):166-74. doi: 10.1007/s12033-013-9692-2. PMID: 23921698.

[12] Hengen, P. N. (1995). "Purification of His-Tag fusion proteins from Escherichia coli." Trends in Biochemical Sciences 20(7): 285-286. doi:10.1016/S0968-0004(00)89045-3

[13] Andersen, K. R., et al. (2013). "Optimized E. coli expression strain LOBSTR eliminates common contaminants from His-tag purification." Proteins: Structure, Function, and Bioinformatics 81(11): 1857-1861. doi:10.1002/prot.24364

[14] Walls, D., et al. (2017). Protein chromatography, Springer. doi: 10.1007/978-1-60761-913-0

[15] Ravikumar, R.; Chen, L. H.; Jayaraman, P.; Poh, C. L.; Chan, C. C., Chitosan-nickel film based interferometric optical fiber sensor for label-free detection of histidine tagged proteins. Biosensors and Bioelectronics 2018, 99, 578-585. doi: 10.1016/j.bios.2017.08.012

[16] Bornhorst, J. A.; Falke, J. J., Purification of proteins using polyhistidine affinity tags. In Methods in Enzymology, Academic Press: 2000; Vol. 326, pp 245-254. doi: 10.1016/S0076-6879(00)26058-8