Improved structure and enhanced the insecticidal activity of Sip1Aa protein by adding disulfide bonds

Lin Wang
Northeast Agricultural University
Jing Wang
Northeast Agricultural University
Ming-Yue Ding
Northeast Agricultural University
Ji-Guo Gao
Northeast Agricultural University
Rong-mei Liu
Northeast Agricultural University
Hai-Tao Li (lihaitao@neau.edu.cn)
Northeast Agricultural University

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Abstract

Sip1Aa is an insecticidal protein of Bacillus thuringiensis at the secretory stage. It has a strong toxic effect on the members of order Coleoptera. To date, there are few available studies on Sip1Aa protein and the inclusion body problem is serious, and this raises the importance to conduct further studies on Sip1Aa protein. Disulfide bonds, as the only covalent bond on protein side chains, play an important role in the stability and function of the proteins. The tertiary structure of Sip1Aa protein was analyzed by homologous modeling and other bioinformatics methods to predict the conserved domain of Sip1Aa protein. Cysteine used to replace these amino acids by site-directed mutation. Consequently, we were able to successfully construct Sip149-251, Sip153-248, Sip158-243, and Sip178-314. These were exposed to ultraviolet radiation, and we found that Sip153-248 and Sip158-243 were the most stable, followed by Sip149-251 and Sip178-314 when compared with Sip1Aa. After the mutant strain was transferred into Escherichia coli BL21, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to detect the inducible expression products. Approximately 37.6 kDa of proteins that were highly expressed in E. coli. We found no significant change in the insecticidal activity.

1 Introduction

Bacillus thuringiensis is a common gram-positive soil bacterium(Siegel and Bacteria [1], 2000; Ichimatsu [2] et.al.2000; Swiecicka [3] et.al. 2002; Lee [4] et.al. 2003; Raymond B [5] et.al. 2010). Due to its high efficiency and specificity to target pests and non-target biological safety, it has become a widely used biological insecticide for pest control in agriculture and forestry(Tabashnik [6] et al. 2003; Daquila [7] et al. 2021;Whalon and Wingerd [8] 2003). Bt insecticidal proteins are composed of insecticidal crystal proteins (ICPs), vegetative insecticidal proteins (VIPs), and secreted insecticidal protein (Sip).

The stable structure of a protein is the key to its biological activity, and the covalent cross-linked disulfide bond is an important force that maintains the spatial structure. Some studies have shown that the newly introduced disulfide bond can affect the protein folding process. In 1987, Pantoliano [9] et al. selected *Bacillus subtilis* protease sites suitable for the introduction of disulfide bonds by program. They eventually acquired mutants that were more stable than the natural proteins. A study by Binley [10] et al. found that the introduction of a disulfide bond between the C-terminal of gp120 and the immune-dominant fragment of gp41 increased the stability of the intermolecular disulfide bond. The experiment performed by Cuicui [11] et al. effect of disulfide bond concentration on various indexes was studied. Disulfide bonds can help protein folding and consequently affect protein stability. Therefore, researchers hope to enhance the effect of proteins by increasing the number of disulfide bonds. Binley [10] have shown that disulfide bonds play an important role in protein formation despite the absence of disulfide bonds in protein end products. Xu [12] et al. found that protein binding affinity was decreased by eliminating disulfide bonds from the PIIIA structure. Lee [4] have shown that effect of disulfide bond elimination on the signal pathway confirms the importance of disulfide bonds in proteins.Lee [4] have shown that even though the SOS gp140 protein was partially denatured by boiling it in sodium dodecyl

sulfate (SDS), the gp120 subunit was still linked to the extracellular domain of gp41, indicating that the intermolecular disulfide bond was quite stable.

At present,Sameh [13] and Xu [14] studies on ICP and VIP proteins are more in-depth, and more species have been discovered. However, there is little research on Sip, and there is no other report on Sip protein, the important thing is that Donovan [15] found Sip1A and its representation in 2006 and Sha [16] find Sip1Ab gene from a native Bacillus thuringiensis strain QZL38 and its insecticidal activity against Colaphellus bowringi Baly. Donovan [15] et al. first discovered a novel BT secretory protein named Sip1A from the genomic library of strain EG2158D, whose similarity with mtx3 which finded by Rungrod [17] was 46% which finded by Donovan [15]. In 2012, Liu Yanjie [18] et al. amplified Sip1A from wild BT strain QZI26. In 2015, Zhang Jinbo [16] et al. successfully cloned a Sip gene with 1,188-bp size from BT strain DQ 89 (accession number JQ965994) and the similarity with the known Sip1A protein was 87%. The results of the bioassay showed that Sip protein had good toxicity to Coleoptera larva [15], with an LC50 of 1.542 μg/mL [19]. In 2018, Sha Junxue [16] identified a novel Sip gene composed of 1,095 bp and encoded 364 amino acids from the BT strain QZI38. After removing the signal peptide of the first 30 amino acids, a 1,005-bp fragment encoding 334 amino acids was amplified and named Sip1Aa; the sequence analysis showed that Sip1Aa did not contain cysteine.

In this experiment, a pair of amino acid sites, which have the potential to form disulfide bonds, was predicted by the online server Disulfide by Design™. Based on the software, Han [20] et al. introduced disulfide bonds into the structure of lipase to improve the conformational stability and enhance the thermal stability of lipase. In 2020, Zhu Fucheng [21] et al. applied site-directed mutagenesis in the study of improving the activity and stability of the enzyme.This study explored the influence of disulfide bonds on the related functions of Sip1Aa protein to enhance the stability of Sip1Aa protein in *E. coli* and further improve the insecticidal activity of Sip1Aa protein by adding disulfide bonds. Additionally, this study is expected to create a technical strategy for introducing disulfide bonds using various databases and design software to provide new ideas and research methods for subsequent research. This study can meet the needs of new protein molecules and provide corresponding engineering bacteria for future research.

2 Results

2.1 Prediction of disulfide key point

Using an online analysis server (http://swissmodel.expasy.org/), twenty pairs of disulfide bond-forming sites were predicted. Seven pairs of sites were screened out based on the Sip1Aa conserved region, Swiss pdbviewer [22], and Laplace conformation map. A pair of amino acid sites with a spacing of less than 50 bp was excluded, and eight cysteine mutation sites were determined, The pET-Sip1Aa plasmid was used as the template for site-directed mutagenesis, and eight pairs of mutant primers T149C-F/R251C-R, T149C-R/R251C-F, G153C-F/H248C-R, G153C-R/H248C-F, T158C-F/K243C-R, T158C-R/K243C-F, K178C-

F/G314C-R, and K178C-R/G314C-F were used, respectively (the sequences were shown in Primersandaminoacidsequences in the additional materials) for PCR amplification according to the instructions in the site-directed mutagenesis kit, and 5 μL of the PCR products were used for agarose gel electrophoresis detection, and the electrophoresis results were shown in Fig. 1. From the electrophoresis results, it can be seen that the size of the PCR amplification product was consistent with the theoretical size.After homologous recombination, colony PCR was used to select a single colony, and the mutant was sent to Jilin Kumei Biotechnology Co., Ltd. for sequencing. The results showed that all eight sites were successfully mutated into cysteine. Primers and amino acid sequences are detailed in Fig. primers and amino acid sequences in supplementary materials.

2.2 Results and analysis of expression of cysteine mutant

In this experiment, different gradients of inducers were set to induce the mutant protein to obtain the mutant protein with high solubility. Therefore, in this experiment, 0 uL, 1 uL, 10 uL, 30 uL, 50 uL and 100 uL of 1 mol/LIPTG were added to 100 mL of bacterial solution for gradient induction. The results showed that after 100 μL of inducer was added through gradient induction of the mutants, we found that all four mutants had very low protein expression levels. Due to the excessive induction dose, the protein expression became too rapid and the protein did not fold in time. Incomplete folding inclusion bodies were formed in the precipitate leading to low protein content in the supernatant. When 0 uL, 1 uL, 10 uL and 30 uL inducers were added, the protein expression levels were found to be very low, and the E.coli did not fully express the protein due to the too low concentration of inducers. When 50 μL of inducer was added, the four mutants were found to be able to express normally and the solubility of proteins is enhanced, as shown in Fig. 3, which proved that the HAP mutation did not affect the bacterial expression. The bands of the four mutants pointed by the arrow were slightly thicker than those of Sip1Aa, suggesting improvement in protein solubility.

2.3 Detection of newly introduced disulfide bonds

DTNB was used to determine the content of free sulfhydryl in samples by colorimetry. If there are thiol compounds in the system, DTNB will turn to yellow 5-mercapto-2-nitrobenzoic acid. As 5-mercapto-2-nitrobenzoic acid has a maximum absorption at 412 nm, the free sulfhydryl group in the sample can be determined by measuring the absorbance at 412 nm. The absorption spectra of DTNB did not interfere with the determination of the sulfhydryl group. After mixing the samples with DTNB, the absorbance was measured at 412 nm using Sip1Aa as a positive control and cysteine standard samples at different concentrations as a control group. The experimental results showed that the absorbance value of the sample was the same as that of the standard sample without cysteine in the control group, thus indicating that there was no free cysteine in the sample, which proved that the cysteines formed disulfide bonds in a pairwise manner. The three-dimensional structures of the mutant proteins are shown in Fig. 3.

2.4 Optimization of fermentation conditions

In order to optimize the conditions for the expression of Sip1Ac promoter guiding Sip1Aa protein, Sip1Aa strain was inoculated into 2×LB medium, the OD600nm value of the bacterial fluid was measured, and

the growth curve of the strain was plotted as shown in Fig. 4.The OD600nm value of the strain increased exponentially in the first 16 h, was stable from 16 h to 48 h, and began to decrease after 48 h.

2.5 Gradient purification of protein

Since different concentrations of imidazole have a greater impact on the protein purification process during protein purification, it is very necessary to set different gradients in the eluent and binding solution for different imidazole concentrations during protein purification. In this experiment, the imidazole gradient of the binding solution was set at 10 mmol/L, 20 mmol/L, and 40 mmol/L, and the imidazole gradient of the eluent was set at concentrations: 40 mmol/L, 250 mmol/L, and 500 mmol/L. The SDS-PAGE results of the purified protein are shown in Fig. 5. Finally, when the concentration of the binding solution was 40 mmol/L and the concentration of the eluate was 250 mmol/L, it was the best for SDS-PAGE purification.

2.6 Determination of insecticidal activity

The quantitative insecticidal activity of the four purified mutant proteins was determined at six concentration gradients of 50, 20, 10, 5, 1, and 0.1 μg/mL. Uncontaminated organic vegetables were soaked in mutant protein and fed with organic vegetables with mutant protein solution to cultured macaque leaf beetle larvae. The numbers of dead and alive insects were counted after 48 h of culture, and then the median lethal concentration LC50 was calculated. Quantitative insecticidal activity test results are shown in Table 1. The insecticidal activity of the mutants Sip 149–251 and Sip 178–314 did not change significantly. The insecticidal activity of the mutant Sip 153–248 increased by 2.76 times, and that of Sip 158–243 increased by 2.26 times.

Table 1
Determination results of insecticidal activity

Protein	LC_{50} (μg/mL)	95% confidence interval	Regression equation	Standard error
Sip 1Aa	1.696	1.440–2.121	y=-1.392 + 0.821	0.138
Sip 149–251	1.743	1.479–2.192	y=-1.432 + 0.822	0.140
Sip 153–248	0.614	0.426–0.791	y=-0.563 + 0.918	0.108
Sip 158–243	0.751	0.567–0.935	y=-0.675 + 0.898	0.109
Sip 178–314	1.409	1.228–1.661	y=-1.415 + 1.004	0.145

2.7 Protein stability test

The protein was irradiated by ultraviolet light at 254 nm. Proteins with different irradiation times were subjected to SDS-PAGE, and the BSA protein quantitative experiment was used to draw Fig. 6. It can be seen that both the mutant and Sip1Aa proteins decreased under the influence of ultraviolet radiation, but the protein of the disulfide bond mutant showed a relatively slower decline than that of Sip1Aa protein.

Sip153-248 and Sip158-243 proteins were able to resist the influence of ultraviolet radiation, followed by Sip 149–251 and Sip 178–314. At 24 h, Sip1Aa, Sip 149–251, and Sip 178–314 decreased to 90%, Sip153-248 to 92.6%, and Sip158-243 to 92% of the original contents.

2.8 Structural Analysis of Mutation Sites

Structural analysis of the T149, G153, T158, K178, K243, H248, R251 and G314 sites of Sip1Aa protein was performed with the software of PDB Viewer.

The midgut PH of leaf beetles is about 7.8, and after site-directed mutagenesis, all specific mutations in the protein are made to cysteine. Analysis of the activity of the mutant protein revealed that there was no significant change in the insecticidal activity of the mutated Sip149-251 and Sip178-314, and it was speculated that the amino acids at these four sites were the inactive sites of the protein, but the insecticidal activity of the Sip153-248 and Sip158-243 mutants changed greatly, by 2–3 times, the solubility of the insecticidal protein increased, and the stability increased, so we speculated that this site may be the key site for insecticidal activity; and because the PH of the environment in the midgut was alkaline, and the isoelectric point of Cys was about 5.0, therefore, under alkaline conditions, the reason for the increased insecticidal activity may be that the ionization degree of the free residues of the amino acids was greatly enhanced compared with the original, which greatly increased the solubility of the insecticidal protein and increased the dissolved protein, which in turn enhanced the insecticidal effect.

According to the basic physicochemical properties of amino acids, we know that both Sip149-251 and Sip187-134 change from hydrophilic amino acids to hydrophobic amino acids, especially amino acid 251 is an extremely hydrophilic amino acid, while amino acids Sip153-248 and Sip158-243 that are not hydrophobic are mutated to hydrophobic amino acids. Therefore, we speculated that the increased activity may be due to the change from hydrophilic amino acids to hydrophobic amino acids, which are usually located in the interior of the active protein and located in the active site, so that the active site is protected by disulfide bonds so that it is easier to be protected and not easily oxidized and inactivated, and then the stability of the mutant protein is enhanced, resulting in enhanced activity.

Sip149-251 and Sip187-134 were mutated from the original amino acid site without any chemical bonds to now with stable disulfide bonds, and the stability increased, however, the insecticidal activity changed weakly, speculating that the possible reason was that Sip149-251 and Sip187-134 were not key active sites of insecticidal proteins; while Sip153-248 and Sip158-243 were mutated from the original hydrogen bonds to the present disulfide bonds, which made the stability of the structure of insecticidal proteins enhanced, the solubility of proteins increased, inclusions decreased, and the insecticidal activity increased by 2.76-fold. On the one hand, it shows that the disulfide bond formed by cysteine plays an important role in the activity of insecticidal proteins, with increased bond energy and enhanced stability of proteins, and it is speculated that this site may be a key site for insecticidal proteins to have high insecticidal activity, and it shows that site-directed mutagenesis is important for the study of insecticidal activity.

In site-directed mutagenesis, we designed the mutated sites in the non-conserved region to enhance the activity on the basis of ensuring the basic insecticidal activity, and then selected the above eight mutated sites to finally obtain two proteins with high activity. It was also demonstrated that Sip153-248 and Sip158-243 have important effects on the activity of insecticidal proteins. However, the amino acid sites of Sip149–251 and Sip187–134 had little effect on the activity of insecticidal proteins.

3 Discussion

Dimerization is a very common method of protein modification in biology. It is an important regulatory mechanism of protein activity. Proteins usually form complexes through noncovalent bonds such as hydrogen bonds, ionic bonds, van der Waals interactions, and hydrophobic bonds. In 2020, Xiaolong [(23)] et al. study of disulfide bonds in non-covalent bond clusters provides accurate structural parameters and important reference data for theoretical evaluation of more complex systems. In 2020, Bandyopadhyay [(24)] et al. the non-covalent interactions between epinephrine and nitroaromatic compounds were studied. In 2021, Huo [(25)] et al. studies of drugs in inactive macromolecules or nanocomposites by breaking covalent bonds and weak non-covalent bonds.Disulfide bonds are stable covalent bonds formed by the interaction of two cysteine residues. The position of cysteine in the protein space, C-S bond, and rotation angle of the S-S bond all affect the formation of disulfide bonds. Therefore, the selection of a suitable mutation site is the key to the formation of disulfide bonds. As a software specially designed to introduce disulfide bonds, Disulfide by Design™ fully considers the factors affecting the formation of disulfide bonds in proteins. Alan [(26)] finded that after a series of calculation rules, it predicts the amino acid sites that may form disulfide bonds in protein molecules, and the prediction accuracy is 99.4%. Therefore, we combined the conserved structure region of Sip1Aa, Swiss pdbviewer [(22)], and Laplace conformation to design a disulfide bond mutation to study the effect on Sip1Aa-related functions. Finally, we selected four pairs of sites for mutations, introduced new disulfide bonds, successfully express 37.6 kDa soluble protein, indicating that these amino acid residues were replaced by alanine without destroying the advanced structure of the protein. And constructed four mutants named Sip149-251, Sip153-248, Sip158-243, and Sip178-314.

A stable structure is the basis for proteins to perform biological functions. As the only covalent bond between protein side chains, disulfide bonds play an important role in the formation of stable proteins. Ran [(27)] et al. found that cysteine substitution had little effect on the protoxin expression and bipyramidal crystal formation. They initially showed that the cysteine mutant of a hydrophobic protein has great potential for more applications due to its self-assembling properties. Sip protein easily forms inclusion bodies during expression, which makes it difficult to extract the protein. By increasing the number of disulfide bonds, it can be highly expressed, and the amount of insoluble inclusion bodies can be reduced. In Fig. 2, we can see that the band of the mutant, located at the target protein position, is thicker than that of Sip1Aa, which proves that the solubility of Sip protein is increased and provides convenience for subsequent research.The results showed that the insecticidal activities of mutants Sip153-248 and Sip158-243 were increased by 2.76 and 2.26 times, respectively, while the insecticidal activities of Sip149-

251 and Sip178-314 did not change significantly. The biological activity, basic physicochemical properties, and molecular characteristics of the four mutant proteins were studied, and finally two mutant proteins with high insecticidal activity were obtained. After analysis of PH, hydrophobicity, conserved regions and other properties, we obtained two mutants with insecticidal activity. We speculated that the reason why Sip153-248 and Sip158-243 had high insecticidal activity may be that this site is one of the key sites of protein insecticidal activity, the chemical bond changes from the original hydrogen bond to the current disulfide bond, the bond energy changes greatly from the original non-covalent bond to the current covalent bond, and the hydrophobicity also changes from the original hydrophilic amino acid to the hydrophobic amino acid, so that the key active site is no longer in the reductive environment, making the protein stability enhanced; Compared with the original, it is easier to ionize, and then obtain the protein with higher solubility and better activity, however, after cysteine mutation, we found that the insecticidal activity of Sip149-251 and Sip178-314 did not change significantly, therefore, we speculated that Sip149-251 and Sip178-314 may not be the key active sites of this insecticidal protein.Alternatively, protein folding is the result of various thermodynamic interactions.For most proteins, the amide bond connecting the amino acid residue on the main chain is the only covalent bond.For secreted proteins or the extracellular portion of cell surface proteins, there may be other covalent bonds in the form of disulfide bridges between the side chains of cysteine residues because they are not exposed to an intracellular reducing environment.In addition to these cross-links, protein folding is mainly maintained by weak interactions, including hydrophobic interactions, electrostatic forces, van der Waals forces, and non-covalent bonds such as hydrogen bonds.Some of these non-covalent interactions are forces inside the protein and some are forces between the protein and the environment.Through the study of Nilofer [(28)] et al., it is shown that although their individual contributions are very small, they act because of the large number of folded proteins, resulting in the final generation of folded structures that can barely maintain stability.

Sip153-248 and Sip158-243 proteins also showed good stability under ultraviolet light, and the protein content of Sip153-248 and Sip158-243 remained at 92.6% and 92% of the original protein content after 24 h of continuous irradiation under ultraviolet light. Therefore, the significance of introducing disulfide bonds into mutants Sip153-248 and Sip158-243 is highly supported.

To date, the structural research of Sip protein after mutation is still insufficient. We can further analyse the structural information of mutant proteins by x-ray, nuclear magnetic resonance, and other methods. By comparing with the Sip1Aa structure, we can obtain a more accurate theory. In this study, four mutants were obtained by adding cysteine to the *Sip1Aa* gene, which may change the function of the original target gene. This provides a research object for future studies that focus on the effect of disulfide bonds on proteins. In order to find the insecticidal protein with strong insecticidal specificity, it is of great theoretical significance and practical value to determine the amino acid localization of Sip1Aa protein. The differences in individual amino acid residues also lead to differences in the insecticidal activity of Sip1Aa, which provides new ideas and methods for follow-up research and provides materials for further studies on Sip expression, insecticidal mechanism, and related function verification.

4 Materials And Methods

4.1 Materials

4.1.1 Strains and plasmids

Escherichia coli JM109 and BL21(DE3) were used as the host strains for cloning and expression studies. Vectors derived from pET-21b(+) were used for the production of recombinant proteins. Plasmid pET21b containing the wild *Sip1Aa* gene was maintained in the laboratory.

4.1.2 Medium and antibiotic

The medium used was Liquid LB: Tryptone 1%, NaCl 1%, Yeast extract 1%. 100 mg ampicillin was dissolved in 1 mL of sterile water, filtered through a 0.22-μm filter membrane for sterilisation, and diluted to 1:1000.

4.1.3 Enzymes and biochemical reagents

Protein marker was purchased from Takara company (for CHINA),. 2× Taq mix DNA polymerase was purchased from CWBIO company. DNA gel recovery kit was purchased from Axygen Company. The site-directed mutation kit was purchased from Nozam, Nanjing. All other reagents were homemade pure reagents.

4.1.4 Solutions and buffers

PBS buffer (pH 7.4): KH_2PO_4 2 mmol/L, Na_2HPO_4 8 mmol/L, NaCl 136 mmol/L, and KCl 2.6 mmol/L was dissolved in 1 L of deionized water. Binding buffer (pH 7.4): sodium phosphate 20 mmol/L, NaCl 0.5 mol/L, and imidazole 10 mmol/L. Elution buffer (pH 7.4): sodium phosphate 20 mmol/L and NaCl 0.5 mol/L. The imidazole concentration gradient was set at 40 mmol/L, 250 mmol/L, and 500 mmol/L. Cysteine standard solution (1 mmol/L): We accurately weighed 0.017563 g L-cysteine, dissolved it in 1 mL methanol, and added dH2O until total solution reached 100 mL. DTNB standard solution (10 mmol/L): We accurately weighed 0.0198175 g DTNB, prepared 50 mL solution with 50 mmol/L Na_2HPO_4 (pH 7.0), and stored it in a brown bottle. DTNB analytical solution (0.1 mmol/L): DTNB analytical solution was prepared by adding 1 mL of DTNB standard solution and 99 mL of 0.25 mmol/L Tris HCl (pH 8.3) buffer solution.

4.1.5 Tested insects

The standardized leaf beetle was donated by the plant protection research of the Chinese Academy of Agricultural Sciences. The toxicity of the mutant protein to the third instar larvae of simian leaf beetles was determined by leaf immersion method.

4.2 Methods

4.2.1 Prediction of disulfide bond

According to Chi [(29)] et al. the amino acid sequence of Sip1Aa was submitted to the automated protein structure homology-modeling server (Phyre2). (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?

id=index) The resulting 3D model was submitted to the ModRefiner server(http://zhanglab.ccmb.med.umich.Edu/ModRefifiner) for optimization. The DEEP VIEW SWISS—PDB VIEWER software from the EXPASY server was used to visualize(https://www.expasy.org/). Using the online analysis server Disulfide by Design™ (http://cptweb.cpt.wayne.edu/DbD2/), the possible disulfide bond sites in *Sip1Aa* gene sequence were analysed. A pair of amino acid sites located in the conservative domain was found. Additionally, the distance between the two amino acid sites was greater than 50 bp and they were mutated to cysteine to form a disulfide bond.The SWISS - PDB file is in Additional Requirements.Predicting the feasibility of disulfide bond formation sites for materials in the Supplementary Material Fig. disulfide bond prediction

4.2.2 Construction of mutants

Methylated pet21b-Sip1Aa transfers to the competent state of *E. coli* JM109 as a template. The PCR reaction system was 50 μL, including 1 μL of template, 25 μL of 2x Max buffer, 1 μL of dNTP mix (10 mmol/L), and 1 μL of Phanta Max Super-Fidelity DNA polymerase. Based on the example of mutant Sip149-251 construction, fragment A was amplified by T 149C-F and R 251C-R, while fragment B was amplified by R 251C-F and T 149C-R. The PCR procedure began with pre-denaturation at 95°C for 30 s, denaturation at 95°C for 15 s, annealing at 72°C for 30 s at a rate of 60 s/KB lasting for 30 cycles. The elongation phase was terminated at 72°C for 5 min, and 1 μL of DpnI was added to react at 37°C for 2 h in order for digestion to occur. Then, the homologous recombination kit was used to recombine fragments A and B to complete two cysteine mutations. The recombinant product was transformed into competent *E. coli* BL 21 and cultured at 37°C for 12 h. The positive clones were identified using primers Sipa-f/Sipa-R. These were sequenced by Jilin Kumei Biotechnology Co., Ltd., and DNA sequences were analysed by dnaman.

4.2.3 Optimization of fermentation conditions

The recombinant strain cloned and expressed in Escherichia coli was diluted 30 times under the condition of 37℃ and 220 RPM with blank medium as control. The absorbance OD 600 value of the bacteria solution was determined at each culture time, and each treatment was repeated for 3 times. Based on this, the growth curve of the bacteria was drawn.

4.2.4 Expression and extraction of mutant protein

A single colony was selected and inoculated into LB tubes containing ampicillin resistance at 37°C, 220 rpm for 12 h. This was cultured at 220 rpm for 12 h, and inoculated into 100 mL LB liquid medium with 1% inoculum at 37°C. The OD600 at 220 rpm was approximately 0.6. We added IPTG in a gradient fashion (0 μL, 1 μL, 10 μL, 30 μL, 50 μL, and 100 μL) at 16°C and 160 rpm for 12 h. The culture was subjected to 8,000 rpm for 15 min at 4°C. The bacteria were collected and cleaned with PBS buffer, which was repeated three times. Ultrasonic crushing was performed under 12,000 rpm at specified time intervals (86%, 3 s, 3 s, and 10 min). Then, the supernatant was centrifuged at 4°C. Protein expression was detected via SDS-polyacrylamide gel electrophoresis (PAGE).

4.2.5 Protein purification

Since the carrier had a tag, it could be purified using a nickel column. The nickel column was fixed by adding five times the column volume of sterile water, followed by eight times the volume of binding solution. The crude protein was added, followed by eight volumes of binding liquid, and finally eluted in a gradient fashion. The concentrations of imidazole were 40, 250, and 500 mmol/L. In this experiment, the imidazole gradient of the binding solution was set at 10 mmol/L, 20 mmol/L, and 40 mmol/L, and the imidazole gradient of the eluent was set at concentrations: 40 mmol/L, 250 mmol/L, and 500 mmol/L. SDS-PAGE was used to detect protein purification.

4.2.6 Determination of free cysteine by DTNB

The standard cysteine solution was diluted with Tris HCl buffer at 25°C. The concentrations were 0, 0.025, 0.05, 0.1, 0.15, and 0.2 mmol/L, respectively. We took 1 mL of the above concentration solution and added it to 5 mL of DTNB analysis solution, shook well, and allowed to rest for 10 min. The absorbance was measured at 412 nm to generate the standard curve. Next, we took 1 mL of the sample and added it to 5 mL of DTNB analysis solution, shook well, allowed to rest for 10 min, measured the absorbance value at 412 nm, and determined the concentration of free cysteine according to the standard curve.

4.2.7 Determination of insecticidal activity

The protein expressed in *E. coli* pet21b was used as the negative control. It was smeared on fresh Chinese cabbages to determine its toxicity to the leaf beetles. Each setup was inoculated with 16 larvae, and each treatment was repeated three times. We cultured it for 48 h under the following conditions: temperature of 27 ℃, relative humidity of 55% ± 5%, and light-dark period of 14 h/10 h. After 48 h, the mortality rate was determined and LC50 was calculated using probit analysis.

$$\frac{\text{Survival rate of control group} - \text{Survival rate of treatment group}}{\text{Survival rate of control group}} \times 100\%$$

4.2.8 Protein stability under UV irradiation

In this experiment, the mutant protein at a concentration of 100 ng/uL was used for quantification, and the mutant protein was subjected to UV irradiation for 30 min, 1 h, 3 h, 6 h, 14 h, 17 h, 20 h and 24 h. After sampling, the protein before mutation was used as a control, respectively, for SDS-PAGE to observe the sample bands. The target protein was quantitatively analysed using Image J [(30)] .

Declarations

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Authors' contributions

Conceptualization, LinW and J-GG; methodology, LinW; software, LinW; validation, LinW, M-YD and JinW; formal analysis, LinW; investigation, LinW; resources, H-TL; data curation, LinW; writing—original draft preparation, LinW; writing—review and editing, H-TL; visualization, LinW and Liu RongMei; supervision, J-GG; project administration, J-GG; funding acquisition, J-GG. All authors have read and agreed to the published version of the manuscript.”

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Conflicts of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

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Data Availability Statement:

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Code availability:

Not applicable

Ethics approval

Not applicable

Consent to participate

All authors have read and agreed to the published version of the manuscript.

Consent for publication

I [Lin Wang] give my consent for information about myself (circle as appropriate) to be published in World Journal of Microbiology and Biotechnology

References

1. Siegel JP. Bacteria. Field Manual of Techniques in Invertebrate Pathology. 2000;197(6):209-30.

2. Ichimatsu T, Mizuki E, Nishimura K, Akao T, Saitoh H, Higuchi K, et al. Occurrence of Bacillus thuringiensis in Fresh Waters of Japan. Current Microbiology. 2000;40(4):217-20.
3. Swiecicka I, Fiedoruk K, Bednarz G. The occurrence and properties of Bacillus thuringiensis isolated from free-living animals. Letters in Applied Microbiology. 2010;34(3):194-8.
4. Lee SM, Wu CK, Plieskatt J, Mcadams DH, Miura K, Ockenhouse C, et al. Assessment of Pfs25 expressed from multiple soluble expression platforms for use as transmission-blocking vaccine candidates. Malaria Journal. 2016;15.
5. Raymond B, Johnston PR, Nielsen-Leroux C, Lereclus D, Crickmore N. Bacillus thuringiensis: an impotent pathogen? Trends in Microbiology. 2010;18(5):189-94.
6. Tabashnik BE, Yves C, Dennehy TJ, Shai M, Sisterson MS, Roush RT, et al. Insect Resistance to Transgenic Bt Crops: Lessons from the Laboratory and Field. Journal of Economic Entomology. 2003(4):4.
7. Daquila BV, Dossi FCA, Moi DA, Moreira DR, Caleffe RRT, Pamphile JA, et al. Bioactivity of Bacillus thuringiensis (Bacillales: Bacillaceae) on Diatraea saccharalis (Lepidoptera: Crambidae) eggs. Pest Management Science. 2021.
8. Whalon ME, Wingerd BA. Bt: Mode of action and use. Archives of Insect Biochemistry and Physiology. 2003;54(4):200-11.
9. Pantoliano MW, Ladner RC, Bryan PN, Rollence ML, Wood JF, Poulos TL. Protein engineering of subtilisin BPN': enhanced stabilization through the introduction of two cysteines to form a disulfide bond. Biochemistry. 1987;26(8):2077-82.
10. Binley JM, Sanders RW, Clas B, Schuelke N, Master A, Guo Y, et al. A Recombinant Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Complex Stabilized by an Intermolecular Disulfide Bond between the gp120 and gp41 Subunits Is an Antigenic Mimic of the Trimeric Virion-Associated Structure. J Virol. 2000;74(2):627-43.
11. Cuicui L, Qiyu L. Effect of disulphide bonds and sulphhydryl concentrations on properties of wheat flour. Czech Journal of Food Sciences. 2020;38(No. 5):265-72.
12. Xu X, Xu Q, Chen F, Shi J, Yu R. Role of the disulfide bond on the structure and activity of μ-conotoxin PIIIA in the inhibition of Na V 1.4. Rsc Advances. 2019;9(2):668-74.
13. Sellami S, Jemli S, Abdelmalek N, Cherif M, Jamoussi K. A novel Vip3Aa16-Cry1Ac chimera toxin: Enhancement of toxicity against Ephestia kuehniella , structural study and molecular docking. International Journal of Biological Macromolecules. 2018;117:752.
14. Xu□ing W, Xin Z, Jiang-tao Y, Zhi□ing W. Effect on transcriptome and metabolome of stacked transgenic maize containing insecticidal cry and glyphosate tolerance epsps genes. Plant Journal. 2018;93(6).
15. Donovan WP, Engleman JT, Donovan JC, Baum JA, Bunkers GJ, Chi DJ, et al. Discovery and characterization of Sip1A: a novel secreted protein from Bacillus thuringiensis with activity against coleopteran larvae. Applied Microbiology and Biotechnology. 2006;72(4):713-9.

16. Sha J, Zhang J, Chi B, Liu R, Gao J. Sip1Ab gene from a native Bacillus thuringiensis strain QZL38 and its insecticidal activity against Colaphellus bowringi Baly. Biocontrol Science and Technology. 2018:1-9.
17. Rungrod A, Tjahaja NK, Soonsanga S, Promdonkoy AB. Bacillus sphaericus Mtx1 and Mtx2 toxins co-expressed in Escherichia coli are synergistic against Aedes aegypti larvae. Biotechnology Letters. 2009.
18. Yanjie L, Haitao L, Rongmei L, Jiguo G. Cloning, expression and bioinformatics analysis of a novel Bt gene Sip(In China). Biotechnology Bulletin. 2012(12):101-5.
19. zhangjinbo, editor Cloning and Bioinformatics Analysis of Sip Gene of Bt Bacteria from Northeast China(In China). National Symposium on Bacillus Young Workers; 2013.
20. Han ZL, Han SY, Zheng SP, Lin Y. Enhancing thermostability of a Rhizomucor miehei lipase by engineering a disulfide bond and displaying on the yeast cell surface. Applied Microbiology and Biotechnology. 2009;85(1):117-26.
21. Zhu F, He B, Gu F, Deng H, Chen N. Improvement in organic solvent resistance and activity of metalloprotease by directed evolution. Journal of Biotechnology. 2019;309.
22. Chi B, Li H, Zhang J, Wei P, Gao J, Liu R. In Silico Structure-Based Identification and Validation of Key Residues of Vip3Aa Involving in Lepidopteran Brush Border Receptor Binding. Applied Biochemistry and Biotechnology. 2018.
23. Ganguly A, Kaza H, Kapoor A, Sheth J, Ali MH, Tripathy D, et al. Comparative Evaluation of the Ostium After External and Nonendoscopic Endonasal Dacryocystorhinostomy Using Image Processing (Matlabs and Image J) Softwares. Ophthalmic Plastic and Reconstructive Surgery. 2017.
24. Marco B, Stefan B, Andrew W, Konstantin A, Gabriel S, Tobias S, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Research. 2014;42(W1).
25. Li X, Lu T, Obenchain DA, Zhang J, Feng G. The Characteristics of Disulfide□entered Hydrogen Bonds. Angewandte Chemie International Edition. 2020.
26. Bandyopadhyay P, Karmakar A, Deb J, Sarkar U, Seikh MM. Non-covalent interactions between epinephrine and nitroaromatic compounds: A DFT study. Spectrochimica Acta Part A Molecular and Biomolecular Spectroscopy. 2019;228:117827.
27. Huo S, Zhao P, Shi Z, Zou M, Yang X, Warszawik E, et al. Mechanochemical bond scission for the activation of drugs. Nature Chemistry. 2021.
28. Dombkowski AA. Disulfide by Design™: a computational method for the rational design of disulfide bonds in proteins. Bioinformatics. 2003(14):1852-3.
29. Ran, Li, Sisi, Yang, Xianfeng, Qiu, et al. The conserved cysteine residues in Bacillus thuringiensis Cry1Ac protoxin are not essential for the bipyramidal crystal formation. Journal of Invertebrate Pathology. 2019.
30. Nilofer C, Sukhwal A, Mohanapriya A, Kangueane P. Protein-protein interfaces are vdW dominant with selective H-bonds and (or) electrostatics towards broad functional specificity. Bioinformation.

2017;13(6):164-73.

Figures

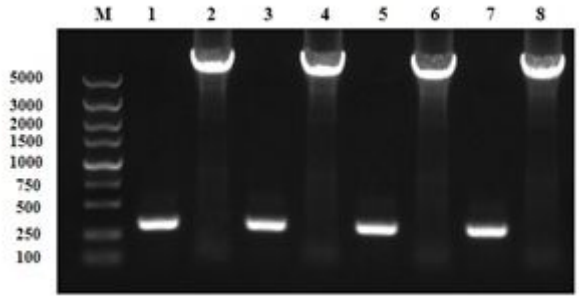

Figure 1

Mutation plasmid PCR amplification electrophoresis detection

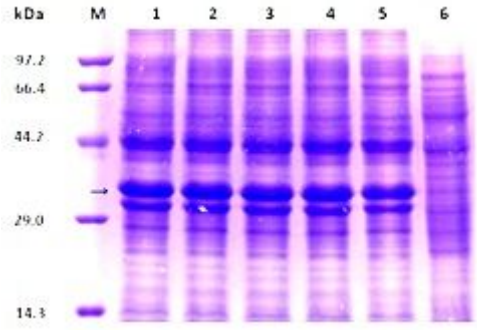

Figure 2

Expression of mutant proteins

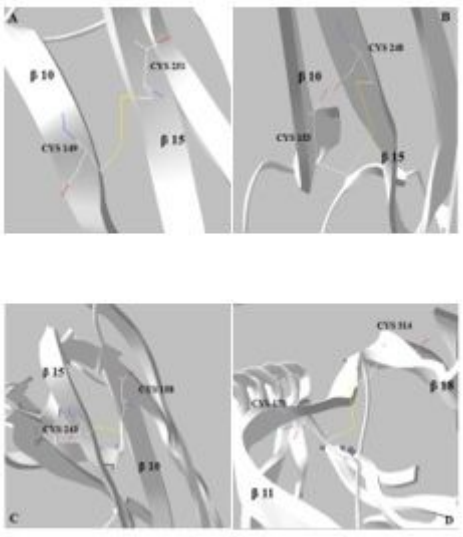

Figure 3

Three-dimensional structures of mutant proteins

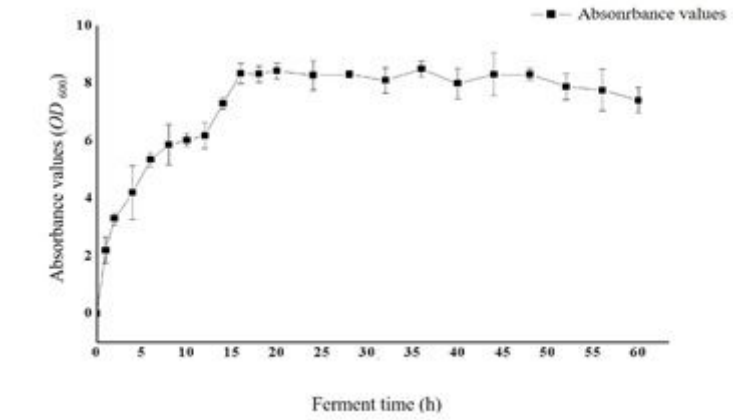

Figure 4

Growth curve of strain Sip1Aa

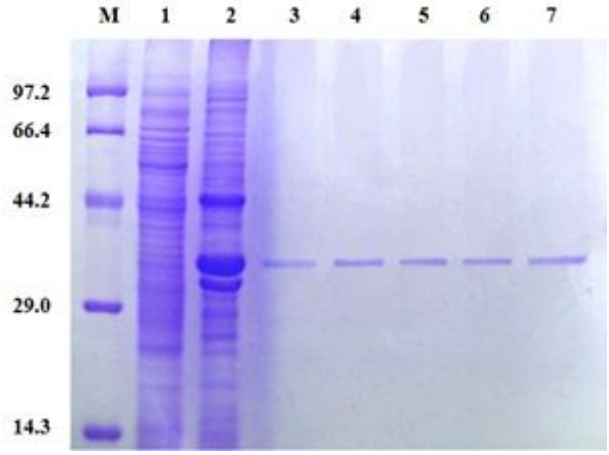

Figure 5

Sip1Aa purification results

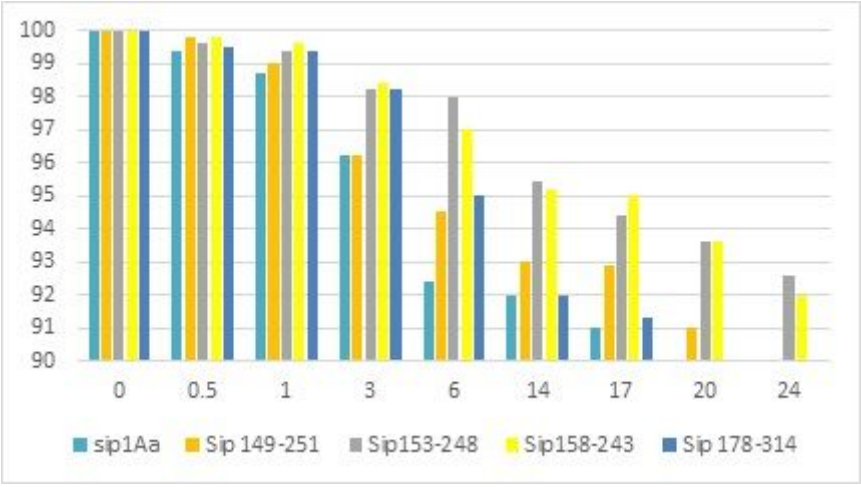

Figure 6

Protein stability