Saturation mutagenesis at Ser196 in BaCsn46A from *Bacillus Amyloliquefaciens* Enhances Enzyme Activity and Thermostability

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Abstract

The chitosanase (BaCsn46A) was extracted from *Bacillus amyloliquefaciens* (GenBank: QEK97559.1) and synthesized after codon optimization. The saturation mutation site was determined by analyzing the sequence and three-dimensional protein model. WT and mutant chitosanase genes were cloned and expressed in *E. coli* BL21 (DE3). The enzymatic properties of WT and mutants were compared, including the optimal reaction pH, temperature and thermostability. Three mutants S196F, S196Y and S196A with the highest specific enzyme activity were selected for further study. Compared with WT, the specific enzyme activity of S196Y increased by 144.76% (more than other two mutants), and the thermostability was not significantly improved. While the specific enzyme activity of S196A increased by 118.79%, and the thermostability of S196A was much higher than WT. From the perspective of industrial production, S196A is more in line with the requirements of industrial production because of its excellent thermal stability at 60°C. From the results of circular dichroism spectrum, the mutation of chitosanase at Ser196 did not change the secondary protein structure. In addition, CD analysis showed that the secondary structure of WT and mutants did not change significantly, indicating that the improvement of thermostability of S196A was not related to the secondary structure.

Introduction

Chitosanase (EC 3.2.1.132) is a glycosyl hydrolase and widely distributed in nature. It has been detected among microorganisms, animals and plants^[1, 2]. According to the current research, the chitosanase activity produced by bacteria is much higher than that of fungi and actinomycetes, and bacteria have the advantage of short fermentation time. By comparison, there is no similarity between the nucleotide sequences of fungal chitosanases and bacterial chitosanases^[3]. According to the similarity of these sequences, chitosanases were categorized to seven glycoside hydrolase (GH) families: GH 2, 5, 7, 8, 46, 75 and 80^[4]. Most of previous reported fungal chitosanases showed significant similarity with each other and are classified as GH 75 family, while the most bacterial chitosanases belong to GH 46 family^[3, 5]. Chitosan is an alkaline polysaccharide. Chitosanase can degrade it into chitooligosaccharides (COS) or glucosamine by hydrolyzing the β -1,4-glycosidic linkage^[6]. Chitosan and COS have strong biological activity, however, the water solubility of chitosan is worse than that of COS, which can only be dissolved in diluted acid, therefore COS plays a greater role in practical application. Based on its non-toxic, antibacterial, degradable characteristics, their practical applications involve various fields such as food, agriculture, medicine and environment^[7]. They have a wide range of functions in wound dressing biomaterials^[8], plant diseases prevention^[9], inhibiting tumor cells^[10], adsorbing of heavy metals in wastewater^[11] and so on.

It was reported that there were many microorganisms with chitosanase production capability through the traditional screening method in the laboratory, such as *Bacillus*^[12, 13], *Pseudomonas*^[14], *Matsuebacter chitosanotabidus*^[15], *Streptomyces*^[16], *Penicillium*^[17], *Chaetomium globosum*^[18], etc. However, the directly selected bacteria have low enzyme activity or low enzyme production efficiency, which can not

meet industrial requirements. Therefore, the biological transformation of the existing chitosanase by means of molecular biology is a good method for improving the enzyme activity and stability. Until now, many chitosanase genes have been cloned and heterologously expressed. *Pichia pastoris* is quite effective in folding exogenous proteins and shows a high capacity of extracellular protein production^[19]. As a protein expression system, *E. coli* is more common than *Pichia pastoris*, and has the advantages of clear genetic background, low cost, high productivity and compatibility with a variety of antibodies^[20].

Directed evolution and rational design can improve the enzymatic properties, such as protein activity and stability, and the recombination of amino acid sequence is the key step of directed evolution and rational design^[21]. As early as the 1970s, Smith^[22] discovered the technology of oligonucleotide site-directed mutagenesis and won the Nobel Chemistry Prize in 1993. This technology also provided new ideas for future generations and greatly promoted the research progress of protein engineering. On the basis of the derived point saturation mutation technology, it can obtain the other 19 amino acids which respectively replace the amino acids at the target site in a short period of time. There are several methods of point saturation mutation, including oligonucleotide directed mutagenesis^[22], cassette mutagenesis^[23], mutagenic oligonucleotide directed PCR amplification^[24], gene spreading by overlap extension and mutagenic plasma amplification^[25].

In our study, *E. coli* was used as a heterologous expression vector. Through the calculation and analysis of gene sequence, we determined the mutation site, and compared the three-dimensional model, enzymatic properties and molecular docking between WT chitosanase and mutant chitosanases. Three mutant chitosanases with higher specific enzyme activity than the WT were obtained in the experiment. Among them, the thermostability of S196Y has been greatly improved, which can have a greater application prospect in industry.

Materials And Methods

Materials

Escherichia coli DH5α, BL21(DE3) were purchased from Takara (Dalian, China) and plasmid pET-28a was purchased from Novagen (Darmstadt, Germany). Chitosan (degree of deacetylation over 80%) and D(+)-glucosamine, hydrochloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Restriction enzymes *Bam*H I and *Hind* III were purchased from Takara (Nanjing, China). The High Purity Plasmid Small Preparation Kit was purchased from Beijing Biotek Corporation Co., Ltd, (Beijing, China). The Site-directed Mutagenesis Kit, the Modified Bradford Protein Assay Kit, Ni-NTA Sefinose(TM) Resin 6FF (Settled Resin) and Desalting Gravity Column were purchased from Sangong Biotech (Shanghai) Co., Ltd. (Shanghai, China). The chitosanase gene was from *Bacillus amyloliquefaciens* (GenBank: QEK97559.1). After optimizing the codon of the gene sequence (BaCsn46A), it was synthesized by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China).

Three-dimensional model construction and homologous sequence comparison of chitosanase

The protein sequence of *Bacillus amyloliquefaciens* (BaCsn46A) was submitted to Swiss-Model (<https://swissmodel.expasy.org/>)^[26] online server to predict its three-dimensional structure, and the chitosanase molecular model from *Bacillus subtilis* MY002 was used as the template (PDB ID : 7C6C. 1. A)^[27]. The three-dimensional model was analyzed on PyMOL 2.4.1 (<https://pymol.org/2/>)^[28]. The amino acid sequence was compared and analyzed by DNAMAN 9.0. The molecular docking was performed between chitosanase and substrate chitotetraose by autodock 4.2 (<http://autodock.scripps.edu/>)^[29].

Design and synthesis of primers

The primers used for saturated site-directed mutagenesis of Ser196 in BaCsn46A were designed and sent to Sangong Biotech (Shanghai) Co., Ltd. (Shanghai, China) for synthesis. The designed primer sequences were shown in Table 1.

Construction of saturated mutation library

The saturated mutation library was constructed by whole plasmid PCR. The PCR system includes Pfu DNA polymerase and dNTP. This process takes plasmid pET-28a as template and the sequences in Table 1 as primers respectively. After PCR, Endonuclease *Dpn* I was added to the product and placed in a 37 °C water bath for 1 h to remove the methylated plasmid. The target plasmid in the PCR product was combined with competent cells made of *E. coli* DH5α and transformed. The above conversion solution which was added to 1 mL Luria-Bertani (LB) solution was incubated at 37 °C and 160 rpm for 1 h. After the bacterial solution was concentrated, it was evenly distributed on the LB solid medium plate containing kanamycin and incubated overnight at 37 °C and 160 rpm. Transformants were selected from the plate and cultured in LB liquid medium. The plasmids were extracted from the bacterial solution according to the kit, and the nucleic acid gel was run to test whether the PCR was successful. Finally, the bacterial solution was sent to Sangong Biotech (Shanghai) Co., Ltd. (Shanghai, China) for sequencing.

Expression and purification of recombinant enzymes

The sequenced recombinant enzymes were reincubated in LB liquid medium at 37 °C and 160 rpm for about 12 hours. Thereafter, 1 % of the above bacterial solution was transferred to a unused LB liquid medium and continued to incubate for 4 hours under the same conditions, and then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to induce protein expression. After being incubated overnight at 16 °C and 160 rpm, the bacterial solution was centrifuged by high speed freezing centrifuge, and the cells were resuspended with buffer M0 (20 mM Tris-HCl solution, 500 mM NaCl solution, 10 % glycerol, pH 8.0, constant volume to 1L). Then, the resuspended solution was ultrasonically broken on ice, centrifuged, and the supernatant was taken out and stored at -20 °C. Ni-NTA column can adsorb proteins with histidine tags and imidazole solutions with different concentration gradients (on the basis of buffer M0, 20-300 mM imidazole solution was added respectively) can be used for elution. Ultimately, the purified chitosanase solution can be stored at -20 °C and used for subsequent determination.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

One volume of protein sample was mixed with 4 times volume of SDS-PAGE loading buffer (2.5 mL 1 M Tris-HCl (pH 6.8), 1 g SDS, 50 mg bromophenol blue, 5 mL glycerol, 0.5 mL β -mercaptoethanol in total). The mixture solution was boiled for 5 min and was added to the 12 % SDS-PAGE gel. The electrophoresis apparatus ran at 80V first, and then changed to run at 120V until the end. After electrophoresis, the gel was dyed and decolorizing. Finally, a picture containing protein bands was obtained on electrophoresis gel imaging system.

Determination of chitosanase activity and protein content

Chitosanase activity was measured by using 3,5-dinitrosalicylic acid (DNS) method with slight modification^[30]. The total reaction system of chitosan enzyme was 2 mL, including 50 mM phosphate buffer (pH 6.6), 1 % colloidal chitosan (chitosan is dissolved in dilute acid, w/v) and an appropriate amount of purified chitosanase solution, which is properly diluted. After being mixed completely, the mixture solution was incubated at 50 °C for 10 min. The reaction was stopped by adding 1.5 mL DNS to the above mixture solution and then the mixture solution was incubated in boiling water for 5 min to develop color. Next, the boiled solution was diluted to 25 mL and centrifuged. Finally, the absorbance of the supernatant was measured at 520 nm with those without enzyme as the control. One enzyme activity unit (U) is defined that the volume of enzyme required for producing 1 μ mol sugar of glucosamine hydrochloride per minute under the experimental conditions (50 °C, reaction for 10 min). The protein content was determined according to the method in the Modified Bradford Protein Assay Kit.

Biochemical characterization of purified chitosanase

The chitosanase activity was determined according to the above method. In order to determine the optimum reaction pH value, the purified chitosanase was measured at 50 °C in different buffer of pH 3-7.2 (citric acid-citrate sodium buffer pH 3-6 and phosphate buffer pH 6-7.2). Under the optimum reaction pH condition, the effect of reaction temperature was studied by measuring the activity of chitosanase at different temperatures ranging from 35 °C to 70 °C. After knowing the optimum reaction pH and temperature, the specific activity of chitosanase can be calculated. The thermostability and pH stability of chitosanase were determined by measuring its activity after being maintained at 60 °C for 90 minutes and on ice at the optimum pH for 8 hours. The enzymatic properties of wild-type (WT, Ser196) and mutants were compared, and then better mutants were selected.

Kinetic parameters assay

Under the optimum reaction conditions, 1 % colloidal chitosan (w/v) was diluted into 1 g/L, 2 g/L, 4 g/L, 6 g/L and 8 g/L, different concentrations of colloidal chitosan were used as substrates for the enzymatic hydrolysis reactions. To ensure the similar hydrolysis degree of the substrate, the reaction time was shortened to 5 min. According to the calculated data, the values of maximum enzymatic reaction rate (V_{max}) and Michaelis constant (K_m) were obtained. The affinity of substrate to chitosanase can be judged from the value of K_m and the catalytic efficiency of chitosanase is determined by the value of K_{cat}/K_m ^[31].

Determination of chitosanase by circular dichroism (CD)

The imidazole in the purified enzyme solution was removed by Desalting Gravity Column. The treated enzyme solutions were sent to Jiangnan University for circular dichroism detection. The circular dichroism results of WT and mutant chitosanases were determined on Chirascan spectropolarimeter (Bio-Logic MOS405, France).

Results

Construction of saturated mutation library

The recombinant chitosanase gene was inserted into plasmid pET-28a and the Ser196 site of the original strain was mutated by site directed mutation PCR. The PCR product eliminated the template plasmid with methylation under the action of *Dpn* I, only the target plasmid was left. The treated PCR product was analyzed by 0.8% agarose electrophoresis, and the result was shown in Fig. 1a. The band was roughly expressed as 6100 bp, which was consistent with that of the target product. The target plasmid was transformed into *E. coli* BL21 (DE3) competent cells, and the saturated mutation library could be obtained by resistance screening and sequencing. The sequencing results can be processed and analyzed by DNAMAN 9.0.

Heterologous expression and enzyme purification

The original strain and mutant strains were heterologously expressed in *E. coli* BL21 (DE3). The chitosanase is an intracellular enzyme, which can be obtained by ultrasonic crushing on ice. The chitosanases were in the supernatant after centrifugation. And the chitosanases were purified through Ni-NTA column. It can be seen from Fig. 1b that the eluent of chitosanase with M300 is almost a single band, indicating that the chitosanase eluted with imidazole solution at this concentration is relatively pure and can be used for subsequent experiments. It can be known that the molecular mass of the chitosanase is about 31 kDa by calculating the mobility (Fig. 1b).

Effect of saturation mutation on activity and stability of chitosanase

The enzymatic properties of 20 amino acids at 196 site were determined respectively, including the optimum reaction pH, temperature and stability. After determining the optimal pH and temperature, the enzyme activity and protein content were measured under these conditions, and the final specific enzyme activity was calculated. Table 2 shows that the specific enzyme activity of three mutant strains were superior to that of the original strain by measuring the enzymatic properties of the original strain and different mutant strains. According to the comparison, the three mutants with the highest specific enzyme activity and wild-type were selected for the following analysis. As shown in Fig. 2A, the enzyme activity measured in phosphate solution as buffer is much higher than that in citrate solution. Ultimately, it was found that the optimum reaction pH of WT and S196A was 6.0, and S196F and S196Y had the highest enzyme activity at pH 6.6 and 7.0, respectively. It is well known that reaction temperature is also a major

factor affecting enzyme activity. Fig. 2B shows that the optimum reaction temperatures of WT, S196F, S196Y and S196A were 55 °C, 60 °C, 45 °C and 55 °C respectively. It can be seen from the figure that S196Y and S196A had higher relative enzyme activities than WT and S196F at low temperature, indicating that they are more suitable for low temperature reaction than the latter. Interestingly, the enzyme activity of S196F reached the highest at 60 °C, while the relative enzyme activity was only about 33 % at 65 °C, indicating that temperature has a great influence on S196F.

It can be seen from Fig. 2C that the enzyme activity of WT, S196F, S196Y and S196A remained at about 100% after being maintained in the buffer with the optimum pH on ice for 8 hours, indicating that the chitosanase has good pH stability. Fig. 2D shows that WT, S196F and S196Y have similar thermostability, while S196A has better thermostability than them. S196A remained 71.31 % and 44.57 % enzyme activity when it was kept at 60 °C for 10 minutes and 30 minutes, respectively. WT, S196F and S196Y kept only 40-50 % and about 15 % at 60 °C for 10 minutes and 30 minutes, respectively. Compared with WT, the specific enzyme activity and thermostability of S196A were improved, which made a certain contribution to the industrial application of chitosanase.

Kinetic analysis of chitosanase

The kinetic parameters corresponding to different amino acids at 196 position after saturation mutation are shown in Table 3. K_m value is inversely proportional to the affinity between enzyme and substrate, and K_{cat}/K_m value is directly proportional to the catalytic efficiency of enzyme. It was measured that the V_{max} of WT was 2426.86 $\mu\text{mol}/\text{min}/\text{mg}$, K_m was 5.31 mg/mL, and K_{cat}/K_m was 236.14 ml/mg/min. Compared to WT, mutants S196F and S196A possessed 1.39- and 1.46-fold increase, respectively, in the K_m value and mutant S196A possessed 1.89- and 1.29-fold increase in the V_{max} and K_{cat}/K_m value.

Results of circular dichroism chromatographic

Circular dichroism (CD) is an important tool to study the thermodynamic stability of proteins. CD provides structural information regarding the bonds and structures based on their chirality^[32]. The scanning of the secondary structure showed that the CD spectrum of the mutant was basically the same as that of WT, indicating that the secondary structure of all mutants was not significantly affected (Fig. 3).

Discussion

Sequence analysis and determination of mutation sites

The sequence of the chitosanase gene (BaCsn46A) extracted from *Bacillus amyloliquefaciens* (GenBank: QEK97559.1) was analyzed based on GenBank database. The gene contained 729 bp, which encoded 242 amino acids. This protein sequence was compared with the protein sequences of other chitosanases in NCBI database, it was found that the chitosanase had 94.9 % identity with other chitosanases from *Bacillus amyloliquefaciens*, 94.36 % identity with chitosanases from *Bacillus subtilis* and 72.27 % identity with chitosanases from *Bacillus cereus*. Multiple sequence alignment of GH46 family members indicated

that the key catalytic residues in BaCsn46A gene are Glu19 and Asp35. According to the previous published study on chitosanase, it is found that these two amino acid residues are the key active sites and they have the characteristics of high conservation^[33]. Once they are mutated into other amino acid residues, the chitosanase activity was greatly reduced, indicating that these two amino acid residues are likely to be the necessary amino acids for catalysis^[34].

Comparing the chitosanase gene sequence produced by different strains (High enzyme activity) with BaCsn46A, it was found that other strains have a certain degree of conservation at Ser196. BaCsn46A is different from them, therefore it was suspected that Ser196 can improve enzyme activity. The three-dimensional model of BaCsn46A was predicted with *Bacillus subtilis* MY002 as the template and the two chitosanases shared sequence identity of 90.50 %. The catalytic residues Glu19 and Asp35 are far away from Ser196 in the amino acid sequence, but they are relatively close in the three-dimensional model after the amino acid was folded. As can be seen in Fig 4, there is a hydrogen bond between the Ser196 and Asp154 of WT and mutant chitosanases. In addition, the Ser196 of mutants have one more bond connected to Pro153 than the WT, which may cause the stability of the mutants to be higher than that of the WT.

Effect of saturation mutation on enzymatic properties

As far as we know, the currently reported highest chitosanase activity in *Bacillus amyloliquefaciens* is *Bacillus amyloliquefaciens* ECU08 (2380.5 U/mg), which was expressed in *Pichia pastoris*^[13]. In our experiment, the specific enzyme activity of WT was 1174.82 U/mg and the highest chitosanase activity of mutants obtained was 1700.62 U/mg. This experiment provides a strong basis for the subsequent saturated site-directed mutagenesis to improve the activity of chitosanase. The optimum pH value of most of the reported chitosanase is 4-7, which is weakly acidic^[35]. The activity of chitosanase isolated from *Bacillus cereus* GU-02 was very stable in the range of pH 7-9, and the optimum pH was 9, but decreased sharply at pH 10^[36]. The chitosanases in our study are basically in accord with the report. The kinetic analysis of the mutants revealed that compared with WT, S196Y had higher affinity and lower catalytic efficiency, S196F had lower affinity and catalytic efficiency, S196A had lower affinity and higher catalytic efficiency. Therefore, we concluded that the conformation of enzymes of different mutants were modified partially at the mutation point, thus affecting the binding of substrate and enzyme. In our experiment, it was found there was little chance that the affinity between enzyme and substrate and catalytic activity of enzyme could be improved at the same time. However, compared with WT, the specific enzyme activity and thermostability of S196A were improved, which made a certain contribution to the industrial application of chitosanase.

Analysis of molecular docking

The docking interaction plots of chitosanase active site and substrate is shown in Fig. 5. The docking sites of WT and mutant chitosanases on the same substrate are slightly different, and the interactions may also change. The Fig. 5 shows that the same seven amino acid residues in WT, S196F, S196Y and

S196A can form nine hydrogen bonds with the substrate, including Glu235, Glu203, Ile145, Gly45, Ala44, Asp35 and Gln146. The Glu19 residue of WT, S196F and S196A form ionic bond with the substrate, while only the Glu19 residue of S196Y form hydrogen bond with the substrate. Generally, the increase of the number of hydrogen bonds can improve the rigidity of the three-dimensional structure and improve the stability, however, the number of unfavorable bumps of S196Y did not decrease. Meanwhile, the figure also shows that the number of unfavorable bumps of S196F and S196A is less than that of WT, indicating that the enzyme active center may bind more closely to the substrate. The change of interaction force between enzyme and substrate may not completely explain the difference of their enzymatic properties.

Bacillus belongs to GH46 family and is the main microbial source for the production of chitosanase. Compared with other glycoside hydrolases, the catalytic tank of GH46 chitosanases show a highly negative charge. Natural chitosan is composed of cationic polysaccharides, which can better combine with chitosanase. This may be the reason for the high activity of chitosanase produced by *Bacillus*^[13, 37]. The isoelectric points of serine, phenylalanine, tyrosine and alanine are all between 5-6. These amino acids are weakly acidic, which may make the substrate and the enzyme molecule combine better, so as to obtain higher enzyme activity or stability. Lyu (2014) emphasize again the importance of acidic residues in the substrate binding cleft^[33].

Analysis of circular dichroism chromatographic

The destruction of the hydrogen bond network of chitosanase CsnA from *Renibacterium* sp. QD1 led to the decline of the thermal properties of the mutants, but did not change the secondary structure of their proteins, indicating that they were independent of the secondary structure^[38], which was consistent with our experimental results.

Conclusion

In this study, the BaCsn46A gene was derived from *Bacillus amyloliquefaciens* (GenBank: QEK97559.1) and synthesized by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China). The mutant chitosanases were expressed in *E. coli* BL21 (DE3) and the molecular weight of purified chitosanase was about 31 kDa by SDS-PAGE. Three better mutant chitosanases were selected according to specific enzyme activity. The results revealed that the optimum reaction pH, temperature and thermal stability of WT and mutant chitosanase were different, which showed that different amino acid residues had a great influence on the reaction conditions, and the number of hydrogen bonds and amino acid hydrophobicity also affected the enzyme activity. In addition, CD analysis showed that the secondary structure of WT and mutants did not change significantly, indicating that the improvement of thermostability of S196A was not related to the secondary structure.

Declarations

Data availability

The gene sequence of chitosanase (BaCsn46A) was from *Bacillus amyloliquefaciens* (GenBank: QEK97559.1). The gene sequences of three mutants with higher enzyme activity were submitted to NCBI (S196F GenBank: OL415164, S196Y GenBank: OL415163, S196A GenBank: OL415165). Other data and material for this article are available upon request.

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Authors Contribution

Wang Yi, Xu Kepan and LuoWen analyzed data and contributed new methods or models. Gao Wenjun and Wang Hailong performed research. Wang Yi wrote the paper. Guo Jing, Hong Tingting and Cai Zhiqiang conceived and designed the study.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no competing interests.

Disclaimer

Any opinions, findings, recommendations, and conclusions in this paper are those of the authors, and do not necessarily reflect the views of Changzhou University.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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Tables

Table 1
Primers used for saturated site-directed mutagenesis of Ser196 in
BaCsn46A

Primer names	Primer sequences (5' to 3')
S196F-F	GACCTGATGAACCCGTTTCGATGAAGATACCC
S196F-R	GGGTATCTTCATCGAACGGGTTTCATCAGGTC
S196L-F	GACCTGATGAACCCGCTCGATGAAGATACCC
S196L-R	GGGTATCTTCATCGAGCGGGTTTCATCAGGTC
S196P-F	GACCTGATGAACCCGCCGGATGAAGATACCC
S196P-R	GGGTATCTTCATCCGGCGGGTTTCATCAGGTC
S196Y-F	GACCTGATGAACCCGTACGATGAAGATACCC
S196Y-R	GGGTATCTTCATCGTACGGGTTTCATCAGGTC
S196C-F	CCTGATGAACCCGTGCGATGAAGATACC
S196C-R	GGTATCTTCATCGCACGGGTTTCATCAGG
S196W-F	GACCTGATGAACCCGTGGGATGAAGATACCC
S196W-R	GGGTATCTTCATCCCACGGGTTTCATCAGGTC
S196H-F	GACCTGATGAACCCGCACGATGAAGATGCCC
S196H-R	GGGTATCTTCATCGTGCGGGTTTCATCAGGTC
S196Q-F	GACCTGATGAACCCGCAAGATGAAGATACCC
S196Q-R	GGGTATCTTCATCTTGCGGGTTTCATCAGGTC
S196R-F	CCTGATGAACCCGAGAGATGAAGATACCCAAG
S196R-R	CTTGGGTATCTTCATCTCTCGGGTTTCATCAGG
S196I-F	CCTGATGAACCCGATCGATGAAGATACCC
S196I-R	GGGTATCTTCATCGATCGGGTTTCATCAGG
S196N-F	CCTGATGAACCCGAACGATGAAGATACCC
S196N-R	GGGTATCTTCATCGTTCGGGTTTCATCAGG
S196K-F	GACCTGATGAACCCGAAGGATGAAGATACCCAAG
S196K-R	CTTGGGTATCTTCATCCTTCGGGTTTCATCAGGTC
S196V-F	GACCTGATGAACCCGGTCGATGAAGATACCC
S196V-R	GGGTATCTTCATCGACCGGGTTTCATCAGGTC

Primer names	Primer sequences (5' to 3')
S196T-F	CCTGATGAACCCGACCGATGAAGATACC
S196T-R	GGTATCTTCATCGGTCGGGTTCATCAGG
S196D-F	GACCTGATGAACCCGGACGATGAAGATACCC
S196D-R	GGGTATCTTCATCGTCCGGGTTCATCAGGTC
S196E-F	GACCTGATGAACCCGGAGGATGAAGATACCC
S196E-R	GGGTATCTTCATCCTCCGGGTTCATCAGGTC
S196M-F	GACCTGATGAACCCGATGGATGAAGATACCCAAG
S196M-R	CTTGGGTATCTTCATCCATCGGGTTCATCAGGTC
S196G-F	CCTGATGAACCCGGGCGATGAAGATAC
S196G-R	GTATCTTCATCGCCCGGGTTCATCAGG

Table 2
Specific activities corresponding to different kinds of amino acid on the site 196 (The enzyme activity was measured at the optimum pH and temperature, and the protein content was measured according to the method of the kit).

Mutant enzyme	Specific activity (U/mg)
WT	1174.82
S196F	1240.95
S196Y	1700.62
S196L	887.19
S196T	468.15
S196P	28.40
S196A	1395.59
S196C	807.00
S196Q	866.06
S196N	1087.27
S196G	1017.45
S196M	784.58
S196I	673.24
S196K	810.98
S196E	735.29
S196H	572.50
S196D	717.50
S196R	406.89
S196V	766.50
S196W	1012.48

Table 3

Kinetic parameters corresponding to different amino acids at site196 after saturation mutation.

Mutant enzyme	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mg/mL)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{mL}/\text{mg}/\text{min}$)
WT	2426.86	5.31	1253.88	236.14
S196F	1779.70	7.40	919.51	124.26
S196Y	764.62	3.58	395.05	110.35
S196L	357.13	5.49	184.52	33.61
S196T	392.58	5.88	202.83	34.50
S196P	35.77	7.10	18.48	2.60
S196A	4584.07	7.75	2368.44	305.60
S196C	2475.40	7.04	1278.96	181.67
S196Q	2426.86	6.04	1253.88	207.60
S196N	1316.70	2.20	680.30	309.23
S196G	4584.07	8.90	2368.44	266.12
S196M	3726.96	8.30	1925.60	232.00
S196I	1490.80	5.59	770.25	137.79
S196K	5640.81	10.83	2914.42	269.11
S196E	13914.01	30.75	7188.91	233.79
S196H	630.98	3.30	326.01	98.79
S196D	876.37	3.33	452.79	135.97
S196R	742.33	4.09	383.54	93.77
S196V	1287.73	3.62	665.33	183.79
S196W	2015.86	5.55	1041.53	187.66

Figures

Figure.

1

Figure 1

Results of electrophoresis. a: PCR products after Dpn I treatment. b: Protein electrophoretogram of chitosanase corresponding to WT and different kinds of mutants. Lane 1, standard protein marker; lane 2, wide-type; lane 3, S196T; lane 4 S196F; lane 5, S196Y; lane 6, S196L; lane 7, S196P; lane 8, S196A; lane 9, S196C; lane 10, S196Q; lane 11, S196N; lane 12, S196G; lane 13, S196M; lane 14, S196I; lane 15, S196K; lane 16, S196E; lane 17, S196H; lane 18, S196D; lane 19, S196R; lane 20, S196V; lane 21, S196W.

Figure. 2

Figure 2

a: Effect of pH on enzyme activity of WT, S196F, S196Y and S196A. Chitosanase was measured at 50 °C in different buffer of pH 3-7.2 (citric acid-citrate sodium buffer pH 3-6 and phosphate buffer pH 6-7.2). b: Effect of temperature (35-70 °C) on enzyme activity of WT, S196F, S196Y and S196A under the optimum pH condition. c: The pH stability of WT, S196F, S196Y and S196A. Chitosanase activity was measured under the optimum conditions after chitosanase was maintained on ice for 8 hours. d: The thermostability of WT, S196F, S196Y and S196A. The activity of chitosanase was measured under the optimum conditions after chitosanase was maintained at 60 °C for 0 min, 10 min, 30 min, 60 min and 90 min.

Figure. 3

Figure 3

Far-UV CD spectra of wild-type and mutants chitosanase in 50 mM PBS (pH 6.0)

Figure. 4

Figure 4

Three dimensional schematic diagram of amino acid hydrogen bonding in chitosanase. a: WT. b: S196F. c: S196Y. d: S196A.

Figure. 5

Figure 5

Molecular docking plan of WT and mutant chitosanases show the interactions between the substrate molecule and the enzyme active center. a: WT. b: S196F. c: S196Y. d: S196A.

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