Title

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Communication

Running title: Directed evolution of a chitosanase

Directed evolution to enhance secretion efficiency and thermostability of chitosanase from *Mitsuaria chitosanitabida* 3001

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Abstract

Chitosanase (ChoA) from *Mitsuaria chitosanitabida* 3001 was successfully evolved with secretion efficiency and thermal stability. The inactive ChoA mutant (G151D) gene was used to mutate by an error-prone PCR technique and mutant genes that restored chitosanase activity were isolated. Two desirable mutants termed M5S and M7T were isolated. Two amino acids Leu74 and Val75 in the signal peptide of ChoA were changed to Gln and Ile, respectively, in the M7T mutant in addition to the G151D mutation. The L74Q/V75I double ChoA mutant showed 1.5 fold higher in specific activity than wild type ChoA due to an efficient secretion of ChoA. One amino acid Asn222 was changed to Ser in the M5S mutant in addition to the G151D mutation. The N222S single ChoA mutant showed 1.2 fold higher in the specific activity and 17% increase of thermal stability at 50ºC than wild type ChoA. This is the first report that succeeded an evolutional increase of enzyme capability among chitosanses.

Keywords

Chitosanase; random mutagenesis; error-prone PCR; thermostability

Chitosanases (EC 3.2.1.132) are glycosyl hydrolases which catalyze the hydrolysis of ß-1,4-glycosidic linkage of chitosan to produce glucosamine oligosaccharides. Chitosan oligosaccharides produced through enzymatic hydrolysis of chitosan are used as food additives, pharmaceuticals, and elicitors in plant cell cultures.[1-3] We previously reported our identification of the chitosanase gene (*choA*) from *Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*) 3001[4], and its product was then classified into family 80 glycosyl hydrolase. [5] We further reported that Glu-121 and Glu-141 are the catalytically important residues of ChoA, [6] and recently reported new chitosan-degrading strains that produced ChoA-like chitosanases.[7] The ChoA protein of *M. chitosanitabida* was efficiently expressed in *Schizosaccharomyces pombe*,[8] which possess chitin synthases and a chitin deacetylase.[9,10]

Enzyme stability is one of the most important properties of industrial enzymes. A series of attempts to enhance enzyme stability by using site-directed mutagenesis, which were based on proposed thermal stabilization mechanisms and three dimensional structure information, have been made.[11,12] However, in the absence of detailed structural information like glycosyl hydrolase family 80 type chitosanse, random mutagenesis combined with selection or screening mutants can be a useful strategy for generating the desired improvement proteins. Simple, rapid and effective screening procedures will be expected to identify the functional mutants of the target enzyme more easily.

Because selection of chitosanase mutants that form larger clear zones than wild type on the chitosan containing plate is difficult to discriminate, we adopted a zero-base screening strategy.[13,14] In this strategy, an inactive type of the mutant gene was on purposely used as a template to start error-prone PCR to obtain a highly active anzyme. Previously we obtained the various activity-negative mutants of chitosanase through the random mutagenesis using hydroxylamine.[6] We chose the G151D mutant of ChoA as a template, because our preliminary investigation on Gly151 residue suggested that this

residue is related to pH and thermal stabilities of ChoA (our unpublished data). We expected the screening of positive chitosanase mutants from the G151D mutant by error-prone PCR would allow us to isolate the mutant that is related to pH and thermal stability. This screening strategy would also make it easier to discriminate the mutants that increased the chitosanase activity.

Here, we show that a secretion level and thermal stability of the ChoA enzyme can be increased by directed evolution and a zero-base screening method.

To generate a mutants library, random mutation was introduced to activity-negative ChoA mutant (G151D) including whole ORF of ChoA by manganese-based error-prone PCR.[15,16] Before to start a large scale screening, the optimal conditions of error-prone PCR for introducing one or two amino acid substitution through the whole ChoA gene (GeneBank accession number: AB010493) were investigated by varying the additional amount of dATP and dGTP (from 0 to 1.0mM) and $MnCl_2$ (from 0 to 1.0mM). We thus obtained the optimized conditions for error-prone PCR as follows; 50μl PCR mixture contained 1 x reaction buffer, 7.0mM $MgCl_2$, 0.2mM $MnCl_2$, 0.25mM dCTP, 0.25mM dTTP, 0.05mM dATP, 0.05mM dGTP, 10ng template DNA, 20pmol forward (5` TCG AGC TCA ATG CAA CTT CCT CGA CCT GAT 3`) and reverse (5` GG CTC GAG TCA CTG CCA CGA CAT GTT 3`) primers, and 2.5 units of Taq polymerase (Toyobo). The amplified *choA* gene included the start and stop codons as indicated by underlines. Sequencing of at least 500 base pairs of 20 randomly picked mutant clones revealed that all mutations were equally distributed throughout the gene and mutation rate was about 0.1%. The reaction was performed using the following program for 30 cycles of 95ºC, 1min; 56ºC, 1min; 72ºC, 2min. The amplified DNA fragments of 1.4kb were purified from an agarose gel using the QIAquick Gel-Purification Kit (Qiagen) and then it was digested with *Sac*I and *Xho*I whose sites were attached with primers. The resulting genes were ligated with the corresponding restriction sites of an expression vector pBlueScript KS(+) and used to transform *E. coli* DH5α by a heat shock method. The *choA* gene was expressed in frame with the *lacZ*

gene in pBluescript KS(+). To construct a mutants library, transformants were plated on Luria-Bertani-chitosan plates containing ampicillin (100μg/ml) and 0.5% colloidal chitosan, then they were grown at 37ºC for two days. Mutant ChoA genes were judged on the basis of the halo forming ability on the plate. Fig.1

We have screened about 10,000 clones for a halo forming ability on the LB-chitosan plate. Among these mutant libraries, we obtained two candidate mutants, termed M5S and M7T, that displayed bigger halos on the LB-chitosan plate than an activity-negative mutant (G151D). Selected mutants were confirmed their halo formation ability on the colloidal chitosan plate (Fig.1A).

DNA sequencing revealed that the M5S mutant possessed one nucleotide mutation of AAC to AGC, that corresponds to Asn to Ser at position 222 in addition to the G151D mutation. M7T mutant showed two nucleotide substitutions CTG to CAG and GTC to ATC resulting in two amino acid changes (L74Q/V75I) in addition to the G151D mutation. Both mutations in the M7T mutant existed in the signal peptide region of ChoA[5].

To analyze the contribution of N222S and L74Q/V75I mutations to wild type enzyme, we restored Asp151 to Gly in M5S or M7T mutants to make a single (N222S) or double (L74Q/V75I) mutants by site-directed mutagenesis. Site-directed mutagenesis at amino acid position 151 of was conducted by using the QuickChange™ site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions with forward (5` G ATC TCC GAC TAC CGC GGC GAC ACC TTC GGT CCG 3`) and reverse (5` CGG ACC GAA GGT GTC GCC GCG GTA GTC GGA GAT C 3`) primers: underlined nucleotides are changed. Constructed mutants were confirmed their chitosanase activities on the colloidal chitosan plate by halo formation. The L74Q/V75I mutant showed a bigger halo than wild type but the N222S did not so much (Fig.1B).

To measure the chitosanase activity, *E. coli* harboring wild type and mutant ChoA genes were cultured in LB medium supplemented with 50μg/ml ampicillin at 37ºC until the A_{600} reached 1.0. Culture solution was centrifuged (950xg, 10min), and Fig.2

then supernatants were used to measure their chitosanase activities using chitosan 8B as a substrate. The specific activities of a N222S single mutant and a L74Q/V75I double mutant were increased than wild type ChoA by 1.2 and 1.5 fold, respectively. As L74Q/V75I mutations are located in its signal sequence, it was speculated that a higher activity of the L74Q/V75I double mutant is due to that the ChoA protein excreted more efficiently (Fig.2A). To compare the excreteion level of the ChoA protein from wild type and mutants, western blot analysis of extra-cellular proteins using an anti-ChoA antibody was undertaken. As it was expected, the L74Q/V75I double mutant showed about 3 times higher level of the ChoA protein than N222S and wild type (Fig.2B). We next determined the optimum temperature and thermal stability of the N222S and L74Q/V75I mutants. Both mutants and wild type enzymes showed maximum activity at 50ºC, but the N222S mutant showed about 30% higher activity than wild type at 50ºC (data not shown). The thermostabilities of the enzymes were estimated by measuring the remaining activities at 30ºC after 1h heat treatment at various temperatures. N222S mutant showed a 17% higher residual activity at 50ºC than wild type but L74Q/V75I showed a similar pattern of thermostability with wild type enzyme (Fig.3). This difference can be repeated observed. We also examined optimum pH and pH stability of mutant enzymes, but there was no alteration comparing to wild type (data not shown).

Fig.3

We have successfully evolved chitosanse from *M. chitosanitabida* 3001 with secretion efficiency and thermostability. A zero-base screening strategy using an activity- negative mutant (G151D) was proven to be useful in our case too. But in spite of a massive screening of mutants, we only got two desirable mutants and their rates of increased activities were not so high (Fig.1A). So, we performed secondary error-prone PCR using M5S mutant (N222S/G151D) and we obtained a couple of mutants that restored their activities. But the result of sequencing of these mutants showed only one common mutation from Asp to Gly at position 151 was found, namely, they returned to an original amino acid of wild type. It may thus indicate that Gly151 is very sensitive to mutation.

We found that the L74Q/V75I double mutant showed in 1.5 fold higher specific activity than wild type with a higher expression level of the ChoA protein. The increase of specific activity is attributed to the 3-fold higher secretion level of ChoA. It has been reported that alteration in the signal peptide affects secretion of mature protein.[18,19] Although many signal peptides sequenced in bacteria chitosanases showed 20-40 amino acid residues in length,[20,21,22] ChoA has a unusual long signal peptide consisting of 80 amino acid residues in length. The result that these L74Q/V75I mutations stimulate the secretion of the ChoA protein from *E. coli,* indicated that our screening strategy also can be applied for optimizing the signal peptide sequence to increase protein secretion in *E. coli*.

We cannot predict what roles of the N222S mutation may play at this moment. There are many different types of interactions within proteins that affect stability, including hydrophobic interaction, hydrogen bonds, electrostatic interactions, and disulfide bonds. It is generally accepted that the enhancement of these interactions leads to an increase in protein stability. Non- enzymatic deamination of Asn in some proteins was reported to destabilize the protein conformation. [23] Substitution of the other amino acid for Asn avoids deamination, thereby allowing to an increase in the thermal stability of proteins. [24,25] The increase in the thermal stability in N222S might be attributed to this type of mechanism.

This is the first report that evolved chitosanase by random mutagenesis. Evolution by error-prone PCR is still a time consuming procedure, but once some desirable mutations were obtained, it contributes to the understanding of structure-function relationships of enzymes. The three-dimensional structure of ChoA, which we are trying to solve, will further inform us a much clearer role of the Ans222 residue and other important residues such as Glu121 and Glu141 we had found[6].

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Fig. 1. Halo formations on the colloidal chitosan plate of *E. coli* expressing ChoA and its mutants. A) Twenty microliters of culture medium containing each *E. coli* DH5α transformant carrying plasmid pBlueScript-G151D or screened mutants (M5S and M7T) were spotted onto the colloidal chitosan plate, followed by incubation for 1 day at 37ºC. B) N222S and L74Q/V75I mutants were confirmed their halo forming activity with wild type ChoA in the same method. Colloidal chitosan plate contains 0.5% colloidal chitosan, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.07% $MgSO_4$, 0.05% NaCl, 0.05% KCl, 0.01% $CaCl_2$, 0.05% yeast extract and 1.5% agar (pH 6.0).

Fig. 2. Chitosanase activities of N222S, L74Q/V75I mutants and wild type ChoA. (A) The chitosanase activity of each culture supernatant. It was determined with colloidal chitosan 8B (80% of deacetylation degree) as a substrate by the modified Schales method.[17] Chitosanase activity was measured as the amount of reducing sugars produced (Filled bars). Protein amount was measured by protein assay kit (Bio Rad) according to the manufacturer's instructions (Open bars). One unit of chitosanase activity was taken as the amount of enzyme that produced 1μmol of reducing sugars per minute. (B) Western blot analysis of ChoA mutant and wild type proteins. 5μg of each protein was loaded in each lane. Anti-ChoA antibody was used for detection. Lane 1, wild type; lane 2, N222S; lane 3, L74Q/V75I. Signals appeared by Western blot analysis were quantified by using the ImageJ analysis software (Version 1.30v; Wayne Rasband, National Institutes of Health, USA).

Fig. 3. Thermostability of chitosanase from N222S, L74Q/V75I mutants and wild type ChoA. To determine thermal stability of chitosanse, the residual activity was measured after the enzyme solution was treated at various temperatures (30–70ºC) for 1h. The values are the average of three measurements. Standard deviation did not exceed 10 percent. Circle, WT; square, N222S; triangle, L74Q/V75I.

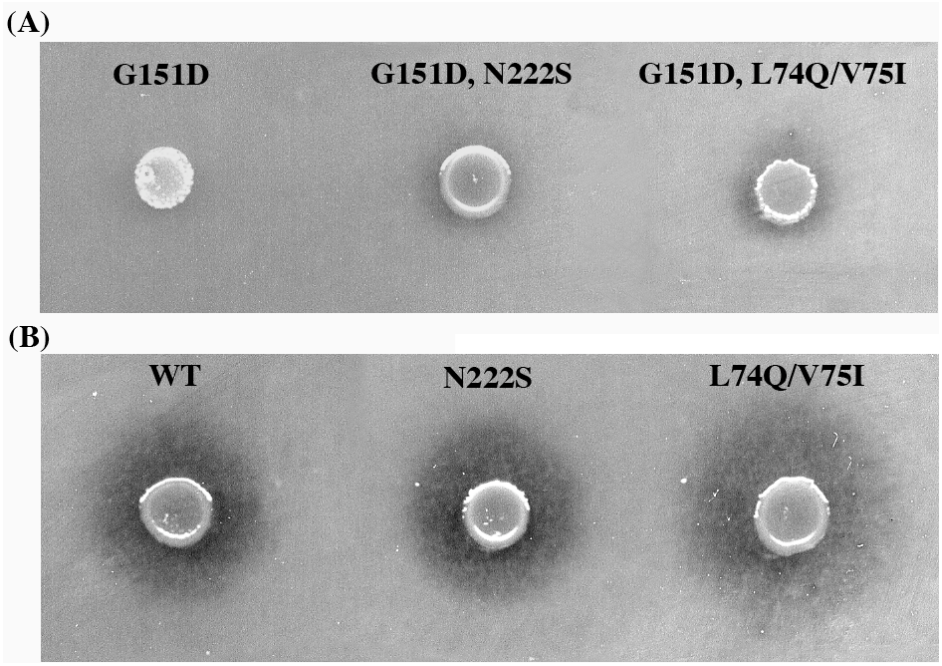
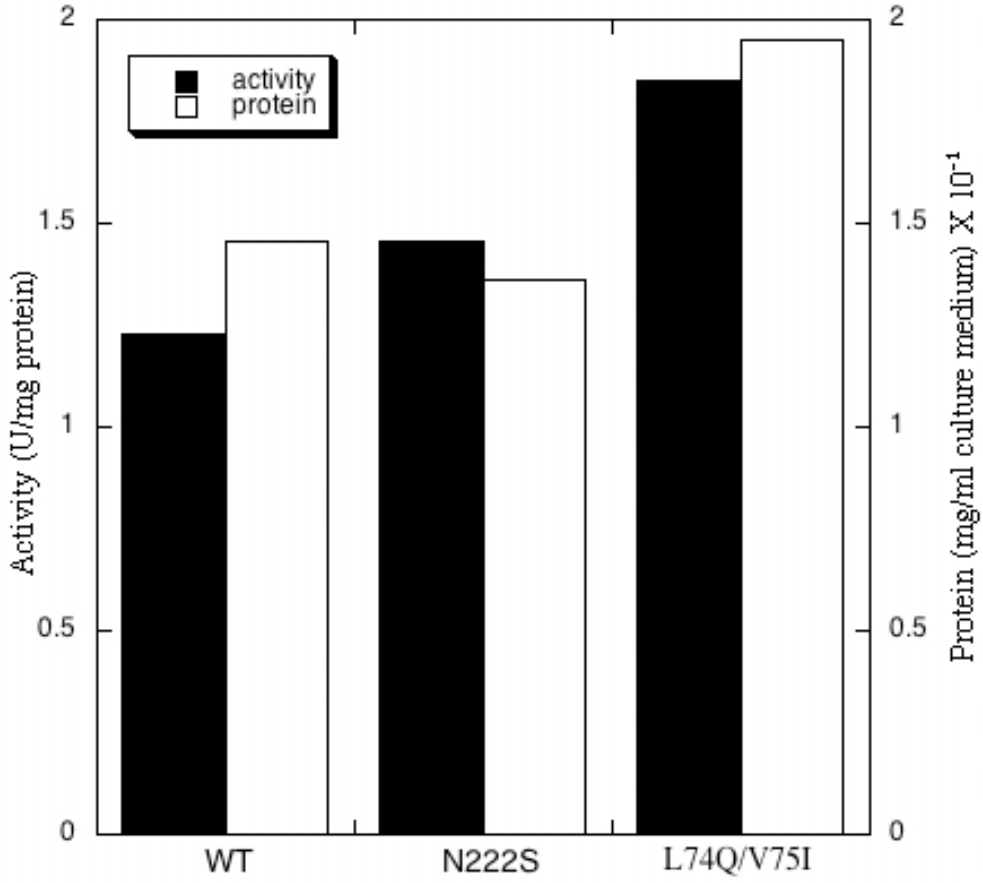


Figure 1. Yun et al.

(A)



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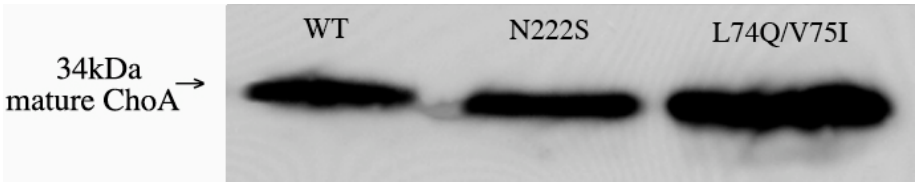

Relative amount	1.00	1.01	1.70

Figure 2. Yun et al.

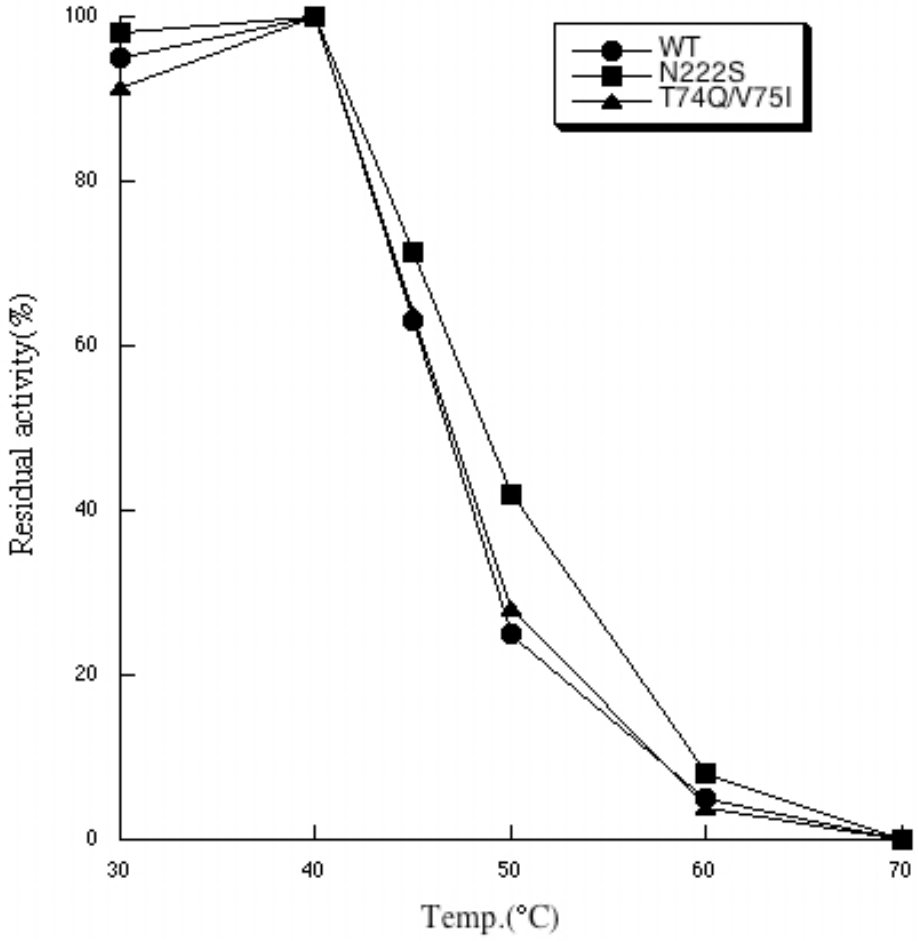

Figure 3. Yun et al.