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学 位 論 文 要 旨
SUMMARY OF DOCTORAL THESIS

氏名 Name: YUN ChoongSoo

題目 Title: Distribution of family 80 type chitosanases in nature and directed evolution of ChoA from *Mitsuaria chitosanitabida*
（自然界における **family 80** キトサナーゼの分布と
Mitsuaria chitosanitabida 由来キトサナーゼ**(ChoA)** の人工進化**)**

Chitin, a poly-ß-1,4 linked *N*-acetyl-ß-D-glucosamine, is widely distributed biopolymers in nature as a major component in the exoskeleton of invertebrates and crustacean and the cell wall of fungi and yeasts. Chitosan, a partially or totally deacetylated derivative of chitin, exist in the cell wall of some fungi and yeasts. Many potential applications have been investigated on the function of chitin, chitosan, and their hydrolyzates, in the field of medicine, biochemistry, pharmacology, enzymology, and agriculture. Especially, low-molecular weight or oligomeric chitin and chitosan have received attention because of their interesting biological properties in agricultural and medical field.

Chitinase (EC 3.2.1.14), which degrades chitin, and chitosanase (EC 3.2.1.132), which degrades chitosan, are widely distributed in living organisms such as bacteria, plants, insects, fungi, and actinomycetes. Various investigations have been conducted on the analysis of structure, function and characterization of chitinase, however, the nature of chitosanase is still poorly understood. To date, many chitosanases have been found in a variety of microorganisms, including bacteria, fungi, plants, and viruses. The chitosanases that have been sequenced so far have been classified into four different families in the classification system of glycosyl hydrolases, namely, Family 8, 46, 75, and 80. This classification of the chitosanases is based on the amino acid sequence similarity of their catalytic domains. Family 8 includes five chitosanases from bacterial organisms along with cellulase, licheninase, and endo-1,4-ß-xylanase. Family 46 includes 18 chitosanases, 16 from bacterial organisms and two from *Chlorella* viruses. The three-dimensional structures of the Family 46 chitosanases from *Streptomyces* sp. N174 and *Bacillus circulans* MH-K1 and the Family 8 chitosanase from *Bacillus* sp. K17 have been determined. The catalytic residues of the Family 8 and 46 chitosanases are reported to be glutamic acid (Glu) and aspartic acid (Asp). Family 75 includes 17 chitosanases, of which 14 and three are from fungi and bacteria, respectively.

Prior to this study, only two bacterial chitosanases have been classified into Family 80. These show no significant nucleotide or amino acid sequence homology with the chitosanases in other families. Chitosanase (ChoA) from *Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*) was the one firstly classified into Family 80. Furthermore, recently it was reported that Glu-121 and Glu-141 are the catalytically important residues of ChoA. Therefore, the author firstly aimed to identify other chitosanases that can be classified into Family 80 and investigated their phylogenetic distribution to determine how commonly this type of chitosanase occurs in nature, and secondly directed evolution of ChoA using random mutagenesis for generating improved chitosanase.

In chapter 1, the author described the general introduction of this thesis.

In chapter 2, the author described the identification and distribution of other chitosanases that can be classified into Family 80 glycosyl hydrolase. To investigate the

phylogenetic distribution of chitosanases analogous to ChoA in nature, the author identified 67 chitosan-degrading strains by screening and investigated their physiological and biological characteristics. The author then searched for similarities to ChoA by Western blotting and Southern hybridization and selected 11 strains whose chitosanases showed the most similarity to ChoA. PCR amplification and sequencing of the chitosanase genes from these strains revealed high deduced amino acid sequence similarities to ChoA ranging from 77% to 99%. Analysis of the 16S rDNA sequences of the 11 selected strains indicated that they are widely distributed in the ß- and γ-subclasses of *Proteobacterium* and the *Flavobacterium* group. These observations suggest that the ChoA-like chitosanases that belong to Family 80 occur widely in a broad variety of bacteria.

In chapter 3, the author reported that directed evolution of chitosanase ChoA. 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.

From the above results, the author concluded two things: 1) Glycosyl hydrolase family 80-type chitosanases occur widely in a broad variety of bacteria in nature. 2) Chitosanase (ChoA) from *Mitsuaria chitosanitabida* 3001 was successfully evolved with secretion efficiency and thermal stability.

These findings contributed to extending our knowledge on chitosanase that belong to glycosyl hydrolase family 80.