CHAPTER IV

DICUSSION

Screening and isolation of bacteria producing chitinase

The bacterium, TU05, was isolated from soil in Pathumwan, Bangkok, Thailand. It can produce clear zone around colony on CCMM plate at 30 °C under aerobic condition within 3 days. This result suggested that, TU05 can produce extracellular chitinase which secreted from cell to degrade chitin when induce by colloidal chitin like some soil-bacteria, such as *Pseudomonas aeruginosa* K-187 [50], *Alteromonas* sp. O-7 [51], *Bacillus circulans* [52], *Streptomyces* sp. No.499 [53], and *Enterobacter* sp. G-1 [55].

Identification of TU05

TU05 was formed round colony with yellow pigments. It was gram positive bacteria, non-motile, non-spore forming, and the morphology of cell was changed during growth like *Microbacterium* sp. which showed the regular rods typical during exponential phase and change to coccoid forms in stationary phase. The biochemical tests of TU05 showed characteristics as same as *Microbacterium* sp. [47]. The 16S rRNA gene sequence of TU05 showed highest similarity to *Microbacterium keratanolyticum* (97%). From all of identification results, TU05 was classified as *Microbacterium* sp. TU05.

Chitinase production of *Microbacterium* sp. TU05

In different medium, 0.2% CCMM and 1% FCMM, showed the different chitinolytic production profile. The chitinase activity was found in

the second day in CCMM and the tenth day in FCMM suggested that both of colloidal chitin and flake chitin can induce chitinase production of *Microbacterium* sp. TU05 as in *Aeromonas hydrophila* H-230 [54]. However, FCMM takes longer time suggested that TU05 might be able to used colloidal chitin easier than flake chitin and grow faster. Then we observed highest chitinolytic activity in less incubation time.

Characterization of crude enzyme from *Microbacterium* sp. TU05

Effect of pH on chitinase activity

After incubation of crude enzyme from *Microbacterium* sp. TU05 in various pHs of the reaction mixture, chitinase activity was observed in broad pH between 4 to 8 (>50% relative activity). The highest chitinase activity was found at pH 5.0 (50.12 mU/ml) and pH 7.0 (42.16 mU/ml). The crude enzyme of *Microbacterium* sp. TU05 had two optimum pHs, this characteristic is like crude chitinase from some bacteria, such as extracellular chitinase from *Pseudomonas aeruginosa* K-187 [50] that had two optimum pHs at pH 7.0 and 8.0, respectively. These results suggested that in crude enzyme from *Microbacterium* sp. TU05 contained two types of chitinolytic enzyme, one had optimum pH at 5.0 and the other had optimum pH at 7.0.

Effect of temperature on chitinase activity

Crude enzyme from *Microbacterium* sp. TU05 was incubated in 0.1M citrate buffer, pH 5.0, and the incubation temperature was varied from 10 to 60 °C. The chitinase activity was observed in range 30 to 50 °C with the optimum temperature at 40 °C (52.451 mU/ml), similar to chitinase from *Aeromonas hydrophila* H-2330 [54].

Substrate specificity

The crude enzyme from *Microbacterium* sp. TU05 had the chitinolytic activity on amorphous chitin, colloidal chitin and regenerated chitin, higher than crystalline chitin, powder chitin and flake chitin. The relative activity of powder chitin and flake chitin compared with colloidal chitin were 35.87% and 11.86%, respectively. In the group of amorphous chitin, the activity on colloidal chitin was higher than regenerated chitin. These results suggested that in crude enzyme contained high exochitinase activity as same as some bacteria, such as *Aeromonas cuviae* [43], *Bacillus licheniformis* [43], and *Xanthomonas* sp. [43]. The crude enzyme has been found hydrolytic activity on chitosan. The activity was decrease when percent deacetylation was increased, but still retains ~50% activity on 90% DD chitosan. This result suggested that there might be some chitosanase activity in crude enzyme. From all of substrate specificity results suggested that *Microbacterium* sp. TU05 secreted both exochitinase and chitosanase in the presence of colloidal chitin as a carbon source. This characteristic of *Microbacterium* sp. TU05 as same as *Enterobacter* sp. G-1 [55] that secreted both chitinolytic and chitosanolytic enzyme in the presence of colloidal chitin or N-acetyl-chitooligosaccharide as a sole carbon sources.

Estimation of chitinase molecular weight

After SDS-PAGE and activity staining, chitinolytic activity of crude enzyme was showed at least two bands with molecular weight 65 and 30 kDa on 10% separating gel. These results suggested that the crude enzyme had at least two species of chitinolytic enzyme. One of chitinolytic enzyme had the molecular weight (65 kDa) close to the molecular weight of chitinase from *Bacillus licheniformis* TP-I (68 kDa) [56] and chitinase from *Vibrio* sp. (63 kDa) [57]. The another one had the molecular weight (30 kDa) close to the

molecular weight of chitinase from various organisms, such as chitinase from Hevamine (29 kDa) [58] and extracellular chitinase FI (30 kDa) and FII (32 kDa) from *Pseudomonas aeruginosa* K-187 [50].

Hydrolysis products of crude enzyme

When the hydrolytic products of crude enzyme, using colloidal chitin as substrate, were analyzed by HPLC. A mixture of products, monomer (*N*-acetylglucosamine) and dimer (*N,N'*-diacetylglucosamine) was obtained, suggesting that crude enzyme contained chitinase and chitobiase activity.

Partial purification of crude enzyme

When proteins in crude enzyme were separated by DEAE cellulose column with 0-1 M NaCl gradient elution, two peaks of protein contained chitinolytic activity were found. After SDS-PAGE and activity staining, the partially purified chitinase peak1 was observed one activity band at 65 kDa, while two activity bands at 55 kDa and 30 kDa were found in partially purified chitinase peak2. From their molecular weight suggested that all of them were chitinase. The molecular weights of chitinases secreted by most of microorganisms were distributed within range of 29 kDa to 115 kDa [55]. The molecular weight of partially purified chitinase peak1 (65 kDa) was close to the chitinase from *Vibrio* sp. (63 kDa) [57]. On the other hand, the partially purified chitinase peak2 had two activity bands, the protein with molecular weight 55 kDa was not found in crude enzyme, but after partial purification, it was obtained in partially purified chitinase peak2. This suggested that in crude enzyme the 55 kDa was a minor protein that was not detected chitinolytic activity on gel, but after purification and concentration, the activity was observed. And the molecular weight of 55 and 30 kDa suggested that both of them were chitinases.

Characterization of partially purified chitinase peak1

The partially purified chitinase peak1 showed the highest activity at pH 5.0 as same as chitinase from *Arthrobacter protophormiae* [I], but remain active in the pH range of 4 to 7 (more than 50% relative activity). These results suggested that the partially purified chitinase peak1 was separated from another chitinase in crude enzyme that had the highest activity at pH 7.0. For optimum temperature, the chitinase activity was observed in range of 30 to 40 °C with the optimum temperature at 40 °C like the chitinase from *Enterobacter* sp. G-1 [55]. These results suggested that the partially purified chitinase peak1 was separated from the other chitinase that had highest activity at higher temperature.

From substrate specificity, the partially purified chitinase peak1 had the highest hydrolytic activity on colloidal chitin. It had the chitinolytic activity on amorphous chitin, colloidal chitin and regenerated chitin, higher than crystalline chitin, powder chitin and flake chitin. The relative activity of powder chitin and flake chitin compared with colloidal chitin were 56.79% and 12.63%, respectively. The activity on colloidal chitin was higher than regenerated chitin and it had no chitosanase activity because the activity was dropped when percent deacetylation of chitosan was increased. These results suggested that the partially purified chitinase peak1 contained mainly exochitinase activity.

DNA Cloning

From shot gun cloning, 30,000 transformants were screened. However, we did not find any positive clones on selective media, suggesting that in chitinase gene of *Microbacterium* sp. TU05 might contained many site of *Pst*I inside them. When DNA was partially digested with *Pst*I, it was cut inside chitinase gene or chitinase gene was not transcribed, translated and processed

to active chitinase or chitinase was produced inside the host cell, but can not be exported into the medium.

Chromosomal DNA of *Microbacterium* sp. TU05 was used for partial chitinase gene amplification. When amplified with primers, designed from conserved amino acid sequence of *Bacillus* sp. family 18 chitinase, the product of 636 bp was obtained. These results suggested that chitinase from *Microbacterium* sp. TU05 was family 18 chitinase. The partially chitinase gene from PCR might be the partially purified chitinase peak1 gene because the partially purified chitinase peak1 was exochitinase with molecular weight (65 kDa). The PCR product from primer BP-I and BP-VI was selected for analyzed by DNA sequencing and compared with the other proteins in GenBank. The results was showed that the partially chitinase gene was highest similarity to chitinase from *Arthrobacter* sp. (71%), which differ from *Microbacterium* sp. TU05 in the family level. *Arthrobacter* sp. was classified in family Micrococcaceae, but *Microbacterium* sp. was classified in family Microbacteriaceae.