CHAPTER 5

GENERAL DISCUSSION

Isolation and characterization of proteolytic enzymes from salt tolerant bacteria

The application of enzymes from halophilic and thermophilic microorganisms as biocatalysis were attractive. Because they were stable and active under stress condition. The enzymes from salt tolerant bacteria and thermophilic bacteria were investigated in this study. Salt tolerant (halotolerant) bacteria were microorganisms that grew in environments of high salt concentration and could be found in hypersaline environments; salt lakes, brines, saline soils and fermented foods products. Proteolytic enzymes from these sources also used in the process of fermentation.

The isolation, screening and characterization of various proteases were studied. We obtained one hundred and eighty two bacteria from various sources in Thailand. Gram positive rods, Gram negative, rods and Gram positive, cocci were found. Liptasiri (1975) found that *Micrococcus, Staphylococcus, Streptococcus, Bacillus , Lactobacillus, Pseudomonas* and *Sarcina* were observed in Thai fish sauce and they required 2-5 % NaCl for growth. *Pediococcus halophilus* was predominant in Thai fish sauce (Jayanadana, 1979). Moderately halophilic bacteria isolated from hypersaline environments in South Spain able to produced different proteases. The optimum growth in media containing 5-15 % salts and up to 20-25 % NaCl salts. These strains were identified as members of the general: *Salinivibrio, Halomonas, Chromohalobacter, Bacillus, Salibacillus, Salinicoccus and Marinococcus* (Porro, *et al*., 2003). In the present study, most of isolated bacteria were moderately thermophilic bacteria that grew maximum at 55 ^{o}C. Most of the isolates could grow

well on medium containing 0-10 % NaCl. They were classified to moderately halophile. The salt concentration at 3-15 % NaCl was the optimal concentration for mederatly halophilic organisms (Ventosa *et al.*, 1995). The primary screening of proteolytic activity were detected on SMA containing various level of NaCl. Zones of clearing were indicative of hydrolysis of protein and thus the production of proteases. Medium containing 0 and 5 % NaCl observed clear zone, but were negative at higher salt concentration. Twelve bacteria showed high protease activity in PY medium. All of them were gram positive, spore forming rods and grew in the presence of 15 % NaCl (2.56 M NaCl). Kushner (1978) defined moderate halophiles as the microorganisms that growing optimally between 0.5 and 2.5 M salt. Bacteria which grew in the absence of salt as well as in the presence of relatively high salt concentrations (8 % in the case of *Staphylococcus aureus*) were designated halotolerant (or extremely halotolerant if growth extends above 2.5 M). A rare case of a bacterium that requires 2 M salt at least (optimal growth at 3.4 M), was exemplified by the actinomycete *Actinopolysora halophila*, was considered a borderline extreme halophile. A moderate halophile could be distinguished from a halotolerant organism by having a requirement for at least 0.1 M NaCl for growth, and they could be seperated from the extreme halophiles by not requiring as much as 1.5 M NaCl for growth. Therefore, in this system the minimum level of NaCl which supported the growth was one of the major parameters. Some halotolerant microbes also required Na^+, but only in the micromolar range. It should be noted that an organism's response to salt depended on other environmental factors (temperature, pH, etc.) (Edwards, 1990).

Calcium chloride in medium had effect on protease production of *Bacillus* sp., K52, K53, K61 and KK128. Glucose 1 % (w/v) and peptone 1 % (w/v) were the best concentration of carbon and nitrogen sources for protease production of *Bacillus licheniformis* K61. *Mycobacterium* sp. produced high level of protease activity in the presence of glucose and sucrose as the carbon sources (Gessesse *et*

al., 1997). A maximum protease activity per cell weight was evident in a defined medium for *B. subtilis* and in a semi-defined and complex media for *S. marcescens*. The richest medium seem to be the best one for protease production (Longo *et al*., 1992). Razak *et al*. (1997) showed that glycerol was the best carbon source, and yeast extract was the best nitrogen source for protease production by *B. stearothermophilus*. Arabinose 1 % and sodium nitrate 1.5 % were the most effective as carbon and nitrogen sources for protease production *Bacillus subtilis* Y-108 (Yang *et al.*, 2000). The four bacteria, K52, K53 and KK128 could be classified to genus *Bacillus* sp., and strain K61 was identified as *Bacillus licheniformis.*

The optimum pH and temperature of crude protease activities from *Bacillus* sp. , K2, K53, K61 and KK128 were at 7.0-8.0 and 50-55 ^{o}C. The enzymes were classified to the neutral metalloprotease family. The proteases in this study showed the optimum pH and temperature similar to the protease from *Bacillus* sp. at pH 7.0-8.0 and 55 ^{o}C (Choorit and Prasertsan, 1992). The crude proteases of the four bacteria investigated were inhibited by EDTA, indicating that these enzyme probably requires a metal ion for either stability or acitivity. The crude proteases from four bacteria were inhibited by Fe^{2+}, Hg^{2+} and Cu^{2+}.

Qua *et al*. (1981) reported that protease from *Pseudomonas* sp. was activated by Mg^{2+}, Co^{2+} and Ca^{2+}, and heavy metal ions such as Fe^{2+}, Cu^{2+} and Hg^{2+} inactivated the enzyme. Thiol reagents and diisoprotpyl fluorophosphate did not affect the enzymatic activity of the protease. The chelating reagent, ethylenediaminetetraacetic acid and endo-phenanthroline, inhibited enzymatic activity, although citrate and oxalate did not affect it. Sharma, *et al*. (1996) reported that protease activity of *Bacillus latersporus* was inhibited by Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions but was enhanced by Ba^{2+}, Ca^{2+} and Fe^{2+} and *Flavobacterium* sp. was inhibited by Mg^{2+} and Mn^{2+} ions but enhanced by Ba^{2+}, Ca^{2+} and Fe^{2+}. The protease activity of *Bacillus subtilis* was activated by Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, but inhibited completely by Hg^{2+}. The protease was also inhibited by metal chelating

agent such as EDTA, sulhydryl reagents as, β-mercaptoethanol, and by cystein hydrocholride, histidine, and glycerol. The EDTA was the most effective inhibitor. This enzyme was a metal-chelator sensitive neutral protease (Yang, *et al.*, 2000). The alkaline serine protease from *Bacillus licheniformis* was activtaed by sodium chloride and stable in presence of 0.7 % $NaBO_3$, 0.5 % NaCl and 3 % H_2O_2 (Manachini and Fortina, 1998). The enzyme from *Bacillus pumilus*, isolated from soybean food was activated by the addition of 1 mM Mn^{2+} and Ca^{2+} ion in the reaction mixture (Yasuda and Aoyama, 2000).

Purification and characterization of an aminopeptidase from *G. thermoleovorans* 47b

The food industry has serious problems concerning the formation of bitter peptides during protein hydrolysis. Debittering of protein hydrolyzates has been carried out by methods involving masking, binding, extraction and enzymes. Debittering enzymes were classified into three groups : aminopeptidases, alkaline/neutral proteases, and carboxypeptidases. The enzymatic hydrolysis of protein was carried out at 60 ^{o}C to prevent microbial contamination. Therefore, to degrade bitter peptides formed during the course of enzymatic hydrolysis of proteins in food processing, we obtained bitter-degrading microbes at 60 ^{o}C from a geothermal field in Oita prefecture using bitter peptides prepared from milk casein as sole carbon and nitrogen source. The isolate 47b that showed leucine aminopeptidase activity in this study was identified as *G. thermoleovorans.*

The purification process of aminopeptidase from *G. thermoleovorans* 47b consisting of seven steps, we homogeneously purified aminopeptidase from *G. thermoleovorans* 47b. Microbial aminopeptidase has been purified and characterized from *Aspergillus sojae* (Chien *et al*., 2002), *A. oryzae* (Nakadai *et al*., 1973), *Aeromonas proteolytica* (Guenet *et al*., 1992), *Alteromonas* B-207 (Merkel *et*

al., 1981), *Bacillus stearothermophilus* (Kuo *et al*., 2003), *Pseudomonas putida* (Hermes *et al*., 1993), *Streptomyces griseus* (Ben-Meir *et al*., 1993), *S. peptidofaciens* (Uwajima *et al*., 1973), *S. rimosus* (Vitale *et al*., 1986), and *S. lividans* (Aphale and Stroh, 1993). Gonzales and Baudouy, 1996 summarized that almost half (47 %) of the 102 bacterial aminopeptidases were monomers, and the remaining (53 %) display a multimeric structure. Leucine aminopeptidase from *P. putida* and *Alteromonas* B- 207 had multimeric structures composed of 8 identical subunits of 53 KDa (Kuo *et al*., 2003), and 2 identical subunits of 33 kDa (Merkel *et al*., 1981), but the enzymes from *Aeromonas* and *Streptomyces* were monomers of 19-32 kDa. Leucine aminopeptidase from *Geobacillus* 47b was a monomer of 42,977 Da. This molecular weight was similar to those reported for *Aeromonas* and *Streptomyces*.

The optimum concentration of carbon and nitrogen sources for aminopeptidase production of *G. thermoleovorans* 47b were 0.5 % meat extract, 0.1 % peptone and 0.01 % yeast extract. The purified aminopeptidase from *G. thermophilus* 47b showed optimum pH and temperature at 7.6 and 60 ^{o}C, respectively. Strains 47 b had an optimal growth temperature at 60 ^{o}C, and produces hyper- thermotolerant leucine aminopeptidase which retains 100 % of its original activity even after 1 h incubation at 90 ^{o}C. The enzyme was strongly inhibited by DTT, suggesting the presence of a disulfide linkage which was required to maintain its active conformation. Thermostability of the enzyme was therefore cause by the presence of a disulfide lingkage. Thermostability of aminopeptidase was due to disulfide bond formation (Chen *et al*., 1997). The aminopeptidase from the thermophillic bacteria, *Pyrococcus horikoshii* was stable at 90 ^{o}C (Ando *et al*., 1999). The aminopeptidase from *Geobacillus thermoleovo-rans* 47b was a thermostable enzyme that considerable potential for many industrial applications. There were potentail industraial needs for such a specific thermostable enzyme, but

improvements were required to maximize their application in the future were also suggested.

Geobacillus 47b was active against Leu-pNA (100 %) and Arg-pNA (56 %), but very low active for Phe-pNA, Lys-pNA, Met-pNA, Ala-pNA, Gly-pNA, Pro-pNA. However, *A. sojae* leucine aminopeptidase was Leu-pNA (100 %), Phe-pNA (98.6 %), Lys-pNA (41.3 %), and Arg-pNA (20.3 %) (Chien *et al*., 2002). *B. stearothermophilus* leucine aminopeptidase was most active against leucine-pNA (100 %) followed by Arg-pNA (46.3 %), Lys-pNA (37.8 %), and Ala-pNA (15.2 %) (Kuo *et al*., 2003). Leucine aminopeptidase from *Thermotoga maritime* was active toward Leu-pNA (100 %), Lys-pNA (68 %, Ala-pNA (27.3 %) and Pro-pNA (7.3 %) (Ratnayake *et al*., 2003). These results indicated that bacterial leucine aminopeptidases had relatively broad substrate specificity. The aminopeptidase from *Bacillus* sp. N_2 (Lee *et al.*, 1998), *Bacillus stearothermophilus* (Kuo *et al.*, 2003), had also high specificity. But the purified aminopeptidase from *Thermus aquatus* YT-1, (Minagawa, *et al.* 1988) and *Lactobacillus plantarum* (Macedo *et al.*, 2003) showed low substrate specificity.

Aminopeptidase could be sub-devided into three groups based on their sensitivity to various inhibitors : metallo-aminopeptidase, cysteine-aminopeptidase and serine-aminopeptidase. We tested several inhibitors of *G. thermoleovarans* 47b enzyme, and found that it was strongly inhibited by bestatin. Its activity was not affected by PMSF and TPCK, classical inhibitors of serine protease and chymotrypsin-like serine protease, and was slightly decreased in the presence of PCMB and N-ethylmaleimide. Enzyme activity was strongly inhibited by EDTA, 1,10-phenanthroline, and N-ethylmaleimide, and dipyridyl, indicating that the enzyme was a metalloenzyme. Leucine aminopeptidase could be classified as a Zn^{2+} metalloenzyme or Co^{2+}-metaloenzyme on the basis of metal requirement. Leucine aminopeptidase from *Aeromonas proteolytica* (Guenet *et al*., 1992), *Alteromonas* B-207 (Merkel *et al*., 1981), *S. griseus* (Ben-Meir *et al*., 1993), *S. peptidofaciens* (Uwajima *et al*., 1973), and *B. kaustophilus* (Lin *et al*., 2004) possess Zn^{2+}, while

leucine aminopeptidase from *B. stearothermophilus* contained Co^{2+} (Myrin and Hofsten, 1974); Stoll *et al*., 1976; Kuo *et al*., 2003). At present, it was not clear which a kind of metal *Geobacillus* 47B leucine aminopeptidase. Simulation of holo-leucine aminopeptidase by Co^{2+} was not observed, and apo-leucine aminopeptidase was reactivated by Co^{2+} but not by Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, indicating that *Geobacillus* 47b leucine aminopeptidase was a Co-dependent aminopeptidase. The crude and purified aminopeptidase in of *G. thermoleovorans* 47b was inhibited by a high concentration of NaCl.

The purified aminopeptidase in this study showed the characteristics similar to the leucine aminopeptidase II of *Bacillus stearothermophilus* as follows. The molecular mass was 44.5 kDa by SDS-PAGE. The temperature and pH optima for the purified protein were at 60 ^{o}C and 8.0, respectively. Under the optimal condition, the purified enzyme showed a marked preference for Leu-p-nitroanilide, followed by Arg and Lys-derivatives. The His6-tagged enzyme was stimulated by Co^{2+} ions, but was strongly inhibited by Cu^{2+} and Hg^{2+} and by the chelating agents, DTT and EDTA. The EDTA-treated enzyme could be reactivated with Co^{2+} ions, indicating that it was a cobalt-dependent exopeptidase (Kuo *et al*., 2003).

The cell free extracts from *G. thermoleovorans* 47b cleaved leucine in significant amounts, suggesting that *G. thermoleovorans* 47b produced leucine aminopeptidase.

Suggestions

1. The proteases produced by the bacteria that obtained in this study was not salt tolerant proteases. However, it was possible to use in foods that contained low salt concentration such as pork sausage, somfak and cheese. The bacteria should be tested for toxicity and consumer acceptation before the application in fermented foods.

2. The cost of enzyme production was a major obstacle in the successful application of such proteases in industry. Protease yield have been improved by screening for hyperproducing strains and /or by optimization of the fermentation medium. Strain improvement by eighter conventinal mutagenesis, or recombinant-DNA technology have been useful in improveing the production of protease. Advances in genetic manipulation of microorganisms by site-direct mutagenesis could also be applied. Gene cloning opens new possibilities for the introduction of precise changes, resulting in the production of tailor-made proteases with novel and desirable properties. The advent of techniques for rapid sequencing of cloned DNA had yielded and explosive increase in protease sequence information.

CONCLUSION

In this study, *Bacillus licheniformis* K61 obtained from Ka-Pi (shrimp paste) grew on medium containing 15 % NaCl. The optimal medium for protease production of this strain was 1 % glucose, 1 % peptone, 0.25 % yeast extract and 0.05 % $CaCl_2$. The optimum pH and temperature were at pH 7.0-8.0 and 50-55 ^{o}C, respectively. The crude proteases were stable at 45 ^{o}C for 24 The crude protease were metalloenzyme. Egg albumin was the best substrate for the crude enzyme from *Bacillus licheniformis* K61.

The aminopeptidase from *G. thermoleovorans* 47b had a molecular mass of 42,710 Da based on SDS-PAGE and 46,773 Da by gel filtration and 42,977 Da by mass spectrometry. The maximum activity was at pH 7.6-7.8 and 60 ^{o}C and stable at 90 ^{o}C for 1 h. Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} at 1mM and EDTA, 0-phenanthroline and dipiridyl inhibited the enzyme and activity restored by Co^{2+}. Increasing of the concentration of sodium chloride was inhibitory to the enzyme; L-Leu-p-nitroanilide was the best substrate. *G. thermoleovorans* 47b produced leucine aminopeptidase.