CHAPTER 4

Purification and characterization of aminopeptidase from thermophilic *Geobacillus thermoleovorans* 47b

Introduction

The enzymatic treatment of various food proteins results in a bitter taste due to the formation of low molecular weight peptides composed mainly of hydrophobic amino acids. Thus, the formation of bitter peptides is the most serious problem in the practical use of food protein hydrolyzates (Saha and Hayashi, 2001). The food industry has employed a range of methods to overcome bitterness in hydrolysed proteins such as masking, binding, and limiting hydrolysis. Protein acid hydrolyzate always found the carcinogens 3-chloropropane-1,2-diol and 1,3-dichloro-2-propanol. The formation of carcinogens is the most serious problem in the practical use of protein hydrolyzates.

Recently, consumers have demanded for healthier and more natural products. Therefore, the enzymatice production of amino acids mixtures from proteins using a combination of proteases and peptidases has recently been paid considerable attention in food protein processing industry. However, the enzymatic hydrolysis of proteins results in a bitter peptides taste due to the formation of low molecular weight peptides composed mainly of hydrophobic amino acids (Ishibashi *et al.*, 1987 and Ney, 1979). The use of peptidase enzymes to reduce or eliminate bitterness has increased significantly in recent years. Peptidases remove single or pairs of amino acids from the terminal of a peptide chain: carboxypeptidases acting from the C-terminal and aminopeptidases from the N-terminal.

Aminopeptidases cleave single or pairs of amino acids from the amino terminal of peptide chains. One of the most common aminopeptidases is leucine aminopeptidase. Most of the industrially used heat-stable enzymes were developed on the assumption

that because all chemical reactions proceed faster at higher temperatures, they could be speeded up still further if temperature-stable enzymes were developed. However, thermostable enzymes are required much greater heating to inactive them at the end of the process. At higher temperatures, there is an additional competitive effect of the denaturation of food protein. Further, to reduce risk of microbial contamination during food protein processing, thermostable enzyme with optimal temperature at about 50-60 ^{o}C should be selected. Aminopeptidases are only comercially produced from two sources: lactic acid-producing bacteria and *Aspergillus oryzae*, which are only stable up to 40 and 60 ^{o}C. To develope a thermotolerant debittering enzyme, we obtained the thermophilic bacteria *Geobacillus thermoleovorans* 47b that isolated from hot spring in Japan which supported by Department of Applied Chemistry, Faculty of Engineering, Oita University, Japan. The objective of this study was to purify and characterize the debittering enzyme, aminopeptidase from *G. thermoleovorans* 47b to evalutaed its usefulness and alternative to commercial aminopeptidase in food processing.

MATERIALS AND METHODS

Bacterium 47b

The bacterium 47 b was isolated from a hot spring in Oita Prefecture at 60 ^{o}C using medium containing (%w/v) 0.5 % bitter peptides, 0.02 % yeast extract and 0.01 % $MgSO_4.7H_2O$ (pH 7.0). This strain was identified by 16S rRNA analysis and supported from Department of Applied Chemistry, Faculty of Engineering, Oita University, Japan. The cultivation condition for this bacterium followed the previous experiment of the student of Department of Applied Chemistry, Oita University.

Effect of concentration of meat extract and peptone on aminopeptidase production

G. thermoleovoran 47b was grown in 2,000 ml Erlenmeyer flask containing 1,000 ml of the following four media incubated on rotary shaker at 150 rpm at 60 °C. The cells was harvested at 18 h, and then centrifuged at 9,000 rpm at 4 °C for 20 min. The harvested cells were washed twice with 10 mM KPB, and stored in a freezer at –20 °C until use. The cells were suspended in 10 mM KPB, and then sonicated for 60 sec. The disrupted cells were collected by centrifugation at 9,000 rpm for 20 min at 4 °C. The cell-free extracts obtained were used for assay of aminopeptidase activity.

Table 4.1 The composition of various media

Composition (%w/v)	**Medium**			
	1	**2**	**3**	**4**
Meat extract	-	0.5	1.0	0.5
Peptone	1.0	0.5	-	0.1
Yeast extract	0.01	0.01	0.01	0.01
KH_2PO_4	0.1	0.1	0.1	0.1
K_2HPO_4	0.1	0.1	0.1	0.1
$MgSO_4.7H_2O$	0.01	0.01	0.01	0.01
pH	7.0	7.0	7.0	7.0

- = no added

Time course of *G. thermoleovarans* 47b

G. thermoleovarans 47b was grown at 60 °C in 1,000 ml of the medium containing (% w/v) 0.5 % meat extract, 0.1 % peptone, 0.01 % yeast extract, 0.1 % K_2HPO_4, 0.1 % KH_2PO_4 and 0.01 % $MgSO_4.7H_2O$ (pH 7.0) in a 2,000 ml Erlenmeyer flask on a reciprocal (New Brunswick Scientific) at 150 rpm. The cell was harvested at 0, 8, 12, 16, 18, 20, 24, 32, 36 and 44 h, and then centrifuged at 9,000 rpm at 4 °C for 20 min. The harvested cells were washed twice with 10 mM KPB (pH 6.8) and subsequently suspended in 10 mM KPB, and then stored at –20 °C until use. Cell growth was measured by cell wet weight. Cell-free extracts from washed cells were prepared by sonication. The disrupted cells were collected by centrifugation at 9,000 rpm for 20 min at 4 °C. The cell-free extracts was dialyzed against 10 mM KPB and was used for assay of aminopeptidase activity.

Preparation of the cell-free extracts

For the production of the enzyme, the bacterium was grown at 60 °C for 18 h in 1,000 ml of the medium containing 0.5 % meat extract, 0.1 % peptone, 0.01 % yeast extract 0.1 % K_2HPO_4, 0.1 % KH_2PO_4 and 0.01 % $MgSO_4.7H_2O$ (pH 7.0) in a 2,000 ml Erlenmeyer flask on a reciprocal at 150 rpm. After cultivation the harvested cells were washed twice with 10 mM KPB (pH 6.8) and subsequently suspended in 10 mM KPB, and then stored at –20 °C until use. Cell-free extracts from washed cells were prepared by sonication. The disrupted cells were collected by centrifugation at 9,000 rpm for 20 min at 4 °C. The cell-free extracts was dialyzed against 10 mM KPB and was used for assay of aminopeptidase activity.

Purification of aminopeptidase

All of procedures were performed at 0–4 °C. The cells (45 g wet weight) collected from 16 liters of culture medium were washed twice with 10 mM KPB and then suspended in 225 ml of 10 mM KPB. The suspended cells were disrupted by sonication. The cell debris was removed by centrifugation at 9,000 rpm at 4 °C for 30 min, and the supernatant was used as a cell-free extract. The cell-free extract was dialyzed overnight against 10 mM KPB, and solid ammonium sulfate was added to the enzyme solution to give 50 % saturation. After 1 h, the precipitate was removed, and the active enzyme was precipitated from the supernatant with ammonium sulfate (70 % saturation) and collected by centrifugation. The precipitate was dissolved with 10 mM KPB and dialyzed overnight against 10 mM KPB. The dialyzed enzyme was loaded onto a DEAE-Toyopearl column (3.0X17 cm) (650 M, Tosoh, Tokyo, Japan) previously equilibrated with 10 mM KPB. After washing the column with 10 mM KPB followed by 10 mM KPB containing 50mM NaCl, the active enzyme was eluted with 10 mM KPB containing 100mM NaCl. The active enzyme was pooled, concentrated by ultrafiltration with an Amicon YM-10 membrane (Amicon Inc., Beverly, MA, USA) and dialyzed against 1 mM KPB (pH 6.8). The enzyme was then applied to a hydroxyapatite column (3.0X7.0 cm) (fast flow type, Wako Pure Chemicals, Osaka, Japan) equilibrated with 1 mM KPB. After washing the column with 1 mM KPB and 20 mM KPB (pH 6.8), the active enzyme was eluted with 40 mM KPB (pH 6.8). The enzyme was next concentrated by ultrafiltration and dialyzed against 10 mM KPB. The dialyzed enzyme was then placed on a Mono Q HR 5/5 column equilibrated with 10 mM KPB (FPLC pump system). The column was washed with 10 mM KPB and 10 mM KPB containing 150 mM NaCl. Following this, the enzyme was eluted with a linear 150 to 200 mM gradient in 10 mM KPB. The active fractions were combined, concentrated by ultrafiltration and dialyzed against 10 mM KPB. The dialyzed enzyme was applied to a Superdex 200 column (1.0X30 cm) equilibrated with 10 mM KPB containing 100 mM NaCl and eluted with the same buffer at 0.2 ml/min using an FPLC pump system. The active enzyme was concentrated by ultrafiltration and dialyzed

against 10 mM KPB, and then the dialyzed enzyme was loaded on a second MonoQ HR 5/5 column equilibrated with 10 mM KPB (FPLC pump system). The column was washed with 10 mM KPB and 10 mM KPB containing 160 mM NaCl. The enzyme was next eluted with 165 mM NaCl in 10 mM KPB. The active enzyme was concentrated by ultrafiltration and dialyzed against 1 mM KPB, and the dialyzed enzyme was applied to a Bio-Scale CHT2-I column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 1 mM KPB (FPLC pump system). After the column was washed with 1 mM KPB and 20 mM KPB (pH 6.8), the enzyme was eluted with a linear 20 to 40 mM gradient using a volume of 60 ml. The active enzyme was finally concentrated by ultrafiltration and dialyzed against 10 mM KPB. Homogeneity of the final aminopeptidase preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli.

Aminopeptidase assay

Aminopeptidase activity was spectrophotometrically assayed by measuring p-NA release from L-Leu-p-NA at 60 °C. The standard assay conditions were as follows. The reaction mixture consisted of 0.4 mM L-Leu-p-NA, 50 mM HEPES buffer (pH 7.6), and the enzyme in a final volume of 1.2 ml. After preincubation at 60 °C for 5 min, the reaction was started by the addition of enzyme. The increase in absorbance at 405 nm was monitored for 15 min on a spectrophotometer (Hitachi Model U 3000). One unit of aminopeptidase activity is defined as the amount of the enzyme that catalyzes the release of 1 μmole of p-NA per min under standard conditions. Specific activity was expressed as units per milligram of protein.

Protein determination

The protein was determined by the method of Lowry *et al*. (1951) using crystalline egg albumin as a standard with most column fractions, protein elution patterns were estimated by the 280 nm absorption.

Characterization of the purified aminopeptidase

Molecular weight estimation

To determine the subunit molecular weight of purified enzyme, PAGE was done in vertical slabs (14X13.5 cm) with 12.5 %(w/v) acrylamide in the presence of SDS by the method of Laemmli. Coomassie Brilliant Blue was used to visualize the protein bands. Myosin (212,000 Da), MBP-β-galactosidase (158,194 Da), β-Galactosidase (116,351 Da), Phosphorylase b (97,184 Da), Serum albumin (66,409 Da), Glutamic dehydrogenase (55,561 Da), MBP 2^{*1} (42,710 Da), Lactate dehydrogenase (36,487 Da), Triosephosphate isomerase (26,625 Da), Trypsin inhibitor 20,040-20,167 Da), Lysozyme (14,313 Da), Aprotinin (6,517 Da), Insulin A,B chain (2,340-3,400 Da) were used as standard marker proteins (Sigma). The native molecular weight of purified enzyme was estimated by gel filtration on a Superdex 200 PC High resolution column (3.2 mmX300 mm) (Amersham Biosciences K.K.) (FPLC system) previously equilibrated with 10 mM KPB containing 100 mM NaCl, at 0.1 ml/min. Cytochrome C (12,400 Da), carbonic anhydrase (29,000 Da), β-amylase (66,000 Da), alcohol dehydrogenase (150,000 Da), and albumin (200,000 Da) were used as standards (Sigma). Molecular weight measurement based on MALDI-TOF mass spectrometry was also performed using a Bruker Autoflex spectrometer (Bruker Daltonik, Leipzig, Germany), where protein calibration standard II was used for calibration.

Effect of pH on enzyme activity and pH stability

The optimum pH for aminopeptidase activity was measured under standard assay conditions using various 50 mM buffers and pHs; pH 5.5-7.0 (MES buffer), pH 6.5-7.5 (MOPS buffer), pH 7.0-8.5 (HEPES and KPB buffer), pH 7.5-9.0 (Tris buffer), and pH 9.0-11.0 (CAPS and CHES buffer). The pH stability was tested by preincubating the enzyme with various buffers and various pHs; pH 5.5-7.0 (MES buffer), pH 6.5-7.5 (MOPS buffer), pH 7.0-8.5 (HEPES and KPB buffer), pH 7.5-9.0

(Tris buffer), and pH 9.0-11.0 (CAPS and CHES buffer) for 30 min at 60 °C before residual enzyme activity was measured.

Effect of temperature on enzyme activity and thermostability

Optimal temperature was also measured at various temperatures (30-90 °C) under standard assay conditions. Enzyme thermostability was determined by preincubation of the enzyme in 50 mM HEPES buffer (pH 7.6) at various temperatures (40 -100 °C) for 1 h and then cooling on ice for 5 min followed by using standard assay conditions to measure residual aminopeptidase activity.

Substrate specificity

The substrate specificity of the purified aminopeptidase for various substrates (Leu-pNA, Lys-pNA, Met-pNA, Arg-pNA, Ala-pNA, Pro-pNA, Gly-pNA, Phe-pNA, Arg-Pro-pNA, Gly-Phe-pNA, Gly-Pro-pNA, Val-Ala-pNA, Gly-Arg-pNA, Ala-Ala-Ala-pNA, Ala-Ala-Phe-pNA, Ala-Ala-Leu-pNA, Pyr-Phe-Leu-pNA, Suc-Ala-Ala-Ala-pNA, Suc-Ala-Phe-Ala-pNA, CBZ-Gly-Gleu-Leu-pNA) was examined under standard assay conditions.

Effect of chelating reagents on enzyme activity

The purified aminopeptidase was preincubated at 60 °C for 5 min with EDTA, o-phenantroline and dipyridyl at a final concentration 0.1, 1.0 and 10.0 mM in 50 mM HEPES buffer (pH 7.6). The enzyme activity was assayed at 60 °C under standard assay conditions.

Effect of cations on enzyme activity

After the purified aminopeptidase was dialysed against 10 mM KPB to remove the contaminating cations, the enzyme was preincubated with various cations; NaCl, KCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CuCl_2$, $CoCl_2$, $FeSO_4$, $FeCl_3$ and $NiCl_2$ at 60 °C for 5 min. The activity was assayed under standard assay conditions.

The metal ion requirement of amipopeptidase

The metal ion requirement for the enzyme activity was tested by adding metal ions to the apo-aminopeptidase. After the enzyme was dialyzed against 1-L changes of 10 mM KPB containing 0.1 mM EDTA at 4 °C for 3 h and then was further dialyzed against 10 mM KPB to completely remove EDTA, the apoenzyme obtained was preincubatted with various 0.2 mM cations (Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}) in 50 mM HEPES buffer (pH 7.6) and the enzyme activity was determined under standard assay conditions.

Effect of chemical reagent on enzyme activity

The effect of various chemical reagents on enzyme activity was determined. After preincubating the enzyme with various chemical reagents (p-chloromercuric benzoate, N-ethylmaleimide, dithiothreitol, mercaptoethanol, iodoacetate, ethylmethanesulfonyl fluoride (PMSF) and tosyl-L-phenylalanine chloromethyl ketone (TPCK)) at 60 °C for 5 min in 50 mM HEPES buffer (pH 7.6), the remaining activity was measured under standard assay conditions.

Effect of sodium chloride on enzyme activity

The salt-tolerance of the enzyme was examined by preincubation in the presence of 2-14 % sodium chloride in 50 mM HEPES buffer (pH 7.6) at 60 °C for 5 min. The enzyme activity was determined under standard assay conditions.

Kinetic constants

The steady state kinetic constants for L-Leu-pNA were determined under standard assay conditions as described above except for substrate concentrations. These activity measurements were made for incubating mixtures ranging in substrate concentrations from 0 to 0.4 mM, the Km and the Vmax were obtained from Lineweaver-Burk plot.

Hydrolysis of bitter peptides

Preparation of bitter peptides from milk casein

Milk casein (10 g, Merck, Damstadt, Germany) was dissolved with 40 ml of 0.1N NaOH under mixing at 50–60 °C and then further dissolved with hot distilled water. To the milk casein solution that was obtained, 10 ml of 0.l M KPB (pH 6.8) was added, this was made up to 100 ml with distilled water to give a 10 % milk casein solution. To this, one tenth g of protease (Amano N, Amano Enzyme Inc., Nagoya, Japan) was added and the reaction was performed at 30 °C for 2-3 h until bitter hydrolyzates were obtained. After boiling the reaction mixture for 10 min, the denatured protein was removed by centrifugation and the supernatant solution was used as a 10 % bitter peptides solution.

Preparation of cell-free extracts from *G. thermoleovorans* 47b

The culture medium was centrifuged at 9,000 rpm at 4 °C for 20 min. Harvest cells were washed twice with 10 mM KPB. The cells (1 g, wet weight) were suspended in 5 ml of 10 mM KPB and disrupted by ultrasonic oscillation by pushing for 10 sec and cooling 15 sec. The total sonication time was 60 sec. The disrupted cells were removed by centrifugation at 9,000 rpm for 30 min at 4 °C, and the supernatant obtained was dialyzed against two changes of 10 ml of 10 mM KPB for 4 h. The dialyzed enzyme was used as a cell-free extract.

Hydrolysis of bitter peptides using cell-free extracts and purified enzyme from *G. thermoleovorans* 47b

The enzymatic hydrolysis of bitter peptides was examined using cell-free extracts or purified enzyme. The reaction mixture contained 100 mM HEPES (pH 7.6), 80 µl of bitter peptides solution and enzyme in a total volume of 200 µl. The bitter peptides solution was placed by water in a blank sample. After incubating at 60 °C for

1- 24 h, the reaction was stopped by the addition of 200 µl of 0.25 M NaOH. The protein in the reaction mixture was removed by the addition of 2 % sulfosalicylic acid followed by centrifugation. An amino acid analysis was performed with an amino acid analyzer JLC-300 (JEOL, Tokyo, Japan).

Hydrolysis of bitter peptides using commercial leucine aminopeptidase

The bitter peptides was hydrolyzed at 30 °C for 1-24 h with leucine aminopeptidase (E.C. 3.4.11.2) Type VI (Microsomal from porcine kidney microsomes). The bitter peptides solution was placed by water in a blank sample. An amino acid analysis was performed with an amino acid analyzer JLC-300 (JEOL, Tokyo, Japan).

Acid hydrolysis of bitter peptides solution

Acid hydrolyses (about of bitter peptides) were carried out using a PICO-TAG workstation (Waters, Massachusetts, USA) with 6 N HCl at 105 °C for 48 h. After hydrolysis, 1 ml of deionized water was added to the hydrolyzate and it was then lyophilized. The amino acids were dissolved into 1 ml of deionized water, and an amino acid analysis was performed with an amino acid analyzer JLC-300 (JEOL, Tokyo, Japan).

Standard methionine (TNBS method)

Prepared D-methionine 0.1, 0.5, 1, 2, 3 and 4 mM. Each concentration was take 200 µl and add 100 µl of 0.25 M NaOH. Incubated at 30 °C for 10 min and after that, add 0.125 M $Na_2B_4O_7$ 100 µl. And added 0.2 M TNBS 40 µl incubate at 30 °C for 10 min. After that, add Na_2SO_3.NaH_2PO_4 solution for 800 µl and then measured at absorbance 520 nm.

Results and Discussion

Bacterium 47b

The 16S rRNA of bacterium 47b sequence was 98 % similarity to that of *Geobacillus thermoleovorans*, and the strain 47b tentatively designated as *G. thermoleovorans* 47b. Recently, thermophilic bacilli has been classified into the new genus *Geobacillus*. Therefore, it is also proposed that *Bacillus stearothermophillus, B. thermoleovorans, B. thermocatenulatus, B. kaustaphilus, B. thermoglucosidasius* and *B. thermodenitrificans* be transferred to this new genus.

Efect of the concentrations of meat extract and peptone on aminopeptidase protduction

The effect of concentration of meat extract and peptone on production of the enzyme was examined. It was found that the highest specific aminopeptidase activity (0.31 U/mg) of aminopeptidase was obtained when 0.5 % meat extract and 0.1 % peptone (medium 4) were used. Whereas the specific aminopeptidases activities of aminopeptidase in medium 1, 2 and 3 were 0.20, 0.19 and 0.14 U/mg, respectively (Table 4.2). The medium which contained and absence of meat extract and peptone showed low aminopeptidase activity. The concentration of carbon and nitrgen compounds prominent role in the aminopeptidase production. In this study showed that 0.5 % meat extract and 0.1 % peptone were the best concentration for high yield of aminopeptidase production. The repression of polypeptone and beef extract on aminopeptidase secretion were reported in *Pseudomonas aeruginosa* (Oh *et al*., 2000).

Table 4.2 Effect of the concentrations of meat extract and peptone on the production of aminopeptidase

Medium	Meat extract (%)	Peptone (%)	Specific activity (U/mg)
1	0	1.0	0.20
2	0.5	0.5	0.19
3	1.0	0	0.14
4	0.5	0.1	0.31

Time course of *G. thermoleovarans* 47b

G. thermoleovorans 47b was cultivated in 1000 ml of optimized medium containing 0.5 % meat extract, 0.1 % peptone, 0.01 % yeast extract, 0.1% KH_2PO_4, 0.1 % K_2HPO_4 and 0.01 % $MgSO_4.7H_2O$ (pH 7.0) in 2,000 ml Erlenmeyer flask. It was found that the maximum aminopeptidase activity of 0.37 U/ml was obtained after cultivated at 60 °C for 18 h. The aminopeptidase production of *G. thermoleovorans* 47b relation to increasing cell growth (Figure 4.1). *G. thermoleovorans* 47b produced maximum amnopeptidase nearly exponential phase. The results showed that aminopeptidase production of *G. thermoleovorans* 47b was growth associated.

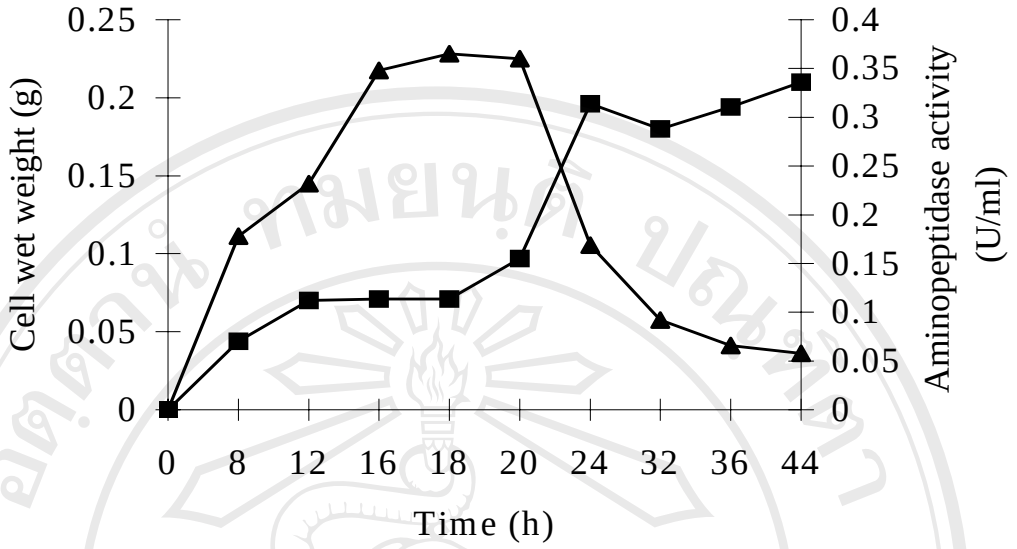

Figure 4.1 Aminopeptidase production (▲) and Cell wet weight (■) in optimized medium of *G. thermoleovorans* 47b the medium volume 1,000 ml of medium 4 contained in 2,000 ml Erlenmeyer flask.

Purification of aminopeptidase

The purification process of aminopeptidase from *G. thermoleovorans* 47b was summarized in Table 4.3. The enzyme was purified and increasing the specific activity in each step. The enzyme was homogenously purified about 164 fold for a 0.01 % yield

Characterization of the purified aminopeptidase

Molecular weight estimation

SDS-PAGE electrophoretogram of preparations obtained after the purification steps were showed in Figure 4.2. A single band was detected after the purification with the second Hydroxyapatite, so the purification process was considered to be completed.

Table 4.3 Purification of aminopeptidase from *G. thermoleovorans* 47b

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extractss	1924	731	0.38	1.0	100
50-70%$(NH4)_2SO_4$	700	273	0.39	1.03	37.3
DEAE- Toyopearl	90.7	122	1.34	3.5	16.6
Hydroxyapatite	8.7	119	13.7	36.1	16.3
Mono Q	0.46	12.2	26.5	70.0	1.67
Gel filtration	0.17	12.2	72.3	190.3	1.66
Mono Q	0.02	1.4	42.7	112.4	0.18
Hydroxyapatite	0.001	0.08	62.6	164.7	0.01

The enzyme was purified about 164 fold. The molecular mass of the purified enzyme was determined to be 42,977 Da by MALDITOF mass spectrometry, which corresponds closely with the relative molecular mass of of the purified enzyme of 46,773 Da determined by Superdex 200 PC gel filtration, and 42,710 kDa found from SDS-PAGE. These results indicated that the native enzyme existed as a monomer.

In addition, the molecular weight of purified aminopeptidase is similar to *Bacillus sterothermophilus.* The molecular mass of aminopeptidase from the other bacteria were showed in Table 4.4.

Effect of pH on enzyme activity and stability

The aminopeptidase activity was determined from pH 5.5-11.0 using different buffer system as described in Materials and Methods. The result was showed in Figure 4.3a. The optimum pH for the purified aminopeptidase was 7.5-9.0, with maximum

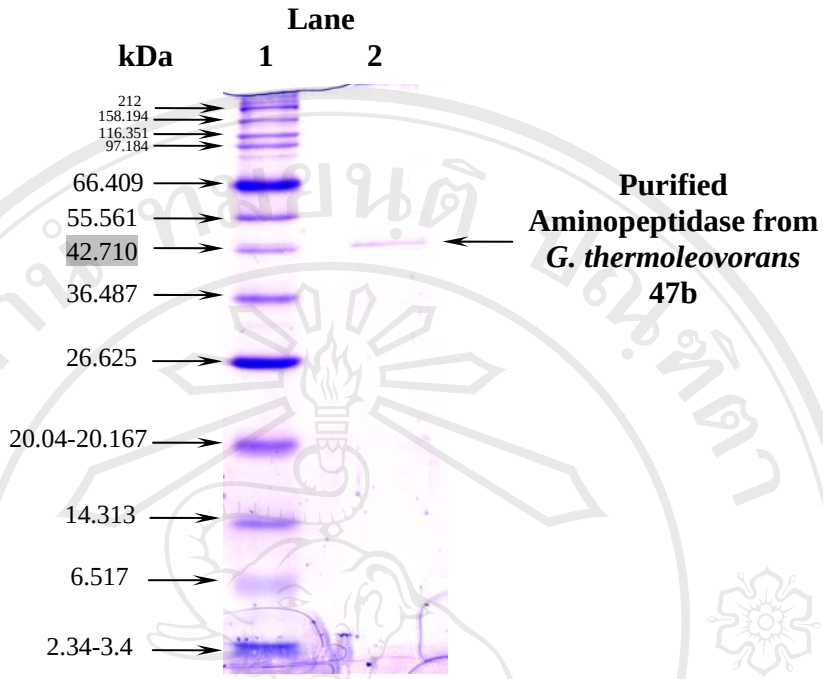

Figure 4.2 Molecular weight estimation of *G. thermoleovorans* 47b aminopeptidase by SDS-PAGE. **Lane 1:** Molecular weight marker; Myosin (212,000 Da), MBP-β-galactosidase(158,194 Da), β-Galactosidase (116,351 Da), Phosphorylase b (97,184 Da), Serum albumin (66,409 Da), Glutamic dehydrogenase (55,561 Da), MBP 2[*1] (42,710 Da), Lactate dehydrogenase (36,487 Da), Triosephosphate isomerase (26,625 Da), Trypsin inhibitor (20,040-20,167 Da), Lysozyme (14,313 Da), Aprotinin (6,517 Da), Insulin A,B chain (2,340-3,400 Da) **Lane 2 :** Purified *G. thermoleovorans* 47b aminopeptidase

activity at pH 7.6. This result was similar to the aminopeptidase from *Lacobacillus sanfrancisco* CB1 reported by Gobbetti *et al*., 1996. However, most of aminopeptidases from various sources were active with the pH range from 7.0-9.5 as summarized in Table 4.4.

The pH stability of the purified aminopeptidase of *G. thermoleovorans* 47b was determined by incubating the purified enzyme at various pHs at 60 °C for 30 min and remaining activities were assayed. The purified aminopeptidase was stable at pH from 7.5-9.0 as shown in the Figure 4.3b. The enzyme was unstable in MES, Tris-HCl, MOPS, KPB, CHES and CAPS buffers.

Table 4.4 Comparison of molecular weight and optimum pH of aminopeptidase from bacteria

Sourses	**MW (KDa)**	**Optimum pH**	**pH stability**	**References**
Lactobacillus sanfrancisco CB1	75	7.5	NR	Gobbetti *et al.*, 1996
Pseudomonas aeruginosa	56	8.5	NR	Canan *et al.*, 2001
Lactobacillus casei subsp. *rhamnosus*	70	7.0	5-9	Arora and Lee, 1994
Streptococcus thermophilus A	95	7.0	NR	Chavagnat *et al.*, 1999
Bacillus sp. N2	58	9.5	NR	Lee *et al.*, 1998
Bacillus kautophilus CCRC11223	53.7	8.0	NR	Lin *et al.*, 2004
Bacillus stearothermophilus	44.5	8.0	NR	Kuo *et al.*, 2003
Geobacillus thermoleovorans 47b	42.7	7.6	7.5-9.0	This study

NR : No result

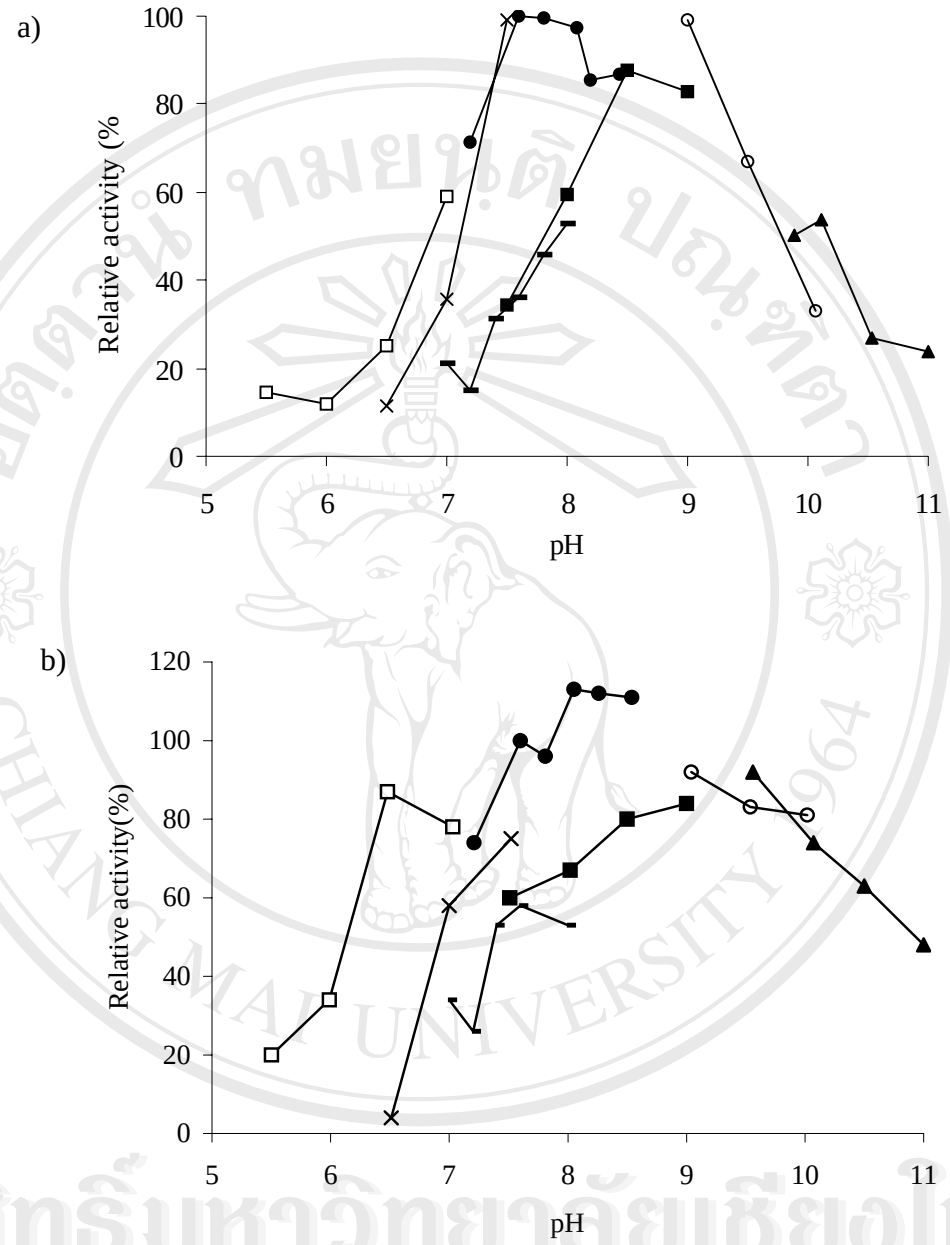

Figure 4.3 Effects of pH on enzyme activity (a) and on enzyme stability (b) MES (□),Tris HCl (■), CAPS (▲), CHES (○), MOPS (×), HEPES (●), KPB (▁)

Effect of temperature on enzyme activity and stability

The effect of temperature (30-90 °C), on the activity of the purified aminopeptidase at pH 7.6 was showed in Figure 4.4. The optimum temperature of purified enzyme was 60 °C, and no aminopeptidase activity at 90 °C. This result was similar to *Bacillus stearothermophilus* (Kuo *et al*., 2003). In contrast with aminopeptidase from *Pyrococcus horikoshii*, thermophillic bacteria showed the optimum temperature over 90 °C (Ando *et al*., 1999). However, the optimum temperature of aminopeptidase for other bacteria was 30-65 °C (Table 4.5).

The effect of temperature on the stability of purified enzyme was showed in Figure 4.5. The enzyme was stable up to 90 °C for 1 h. The aminopeptidase from *G. thermoleovorans* 47b was found to be 100 % stable at 40-90 °C for 1 h, indicating that the aminopeptidase to be highly thermostable. However , the enzyme was no activity at 100 °C. Therefore, the aminopeptidase from *G. thermoleovorans* 47b was classified to thermostable enzyme.

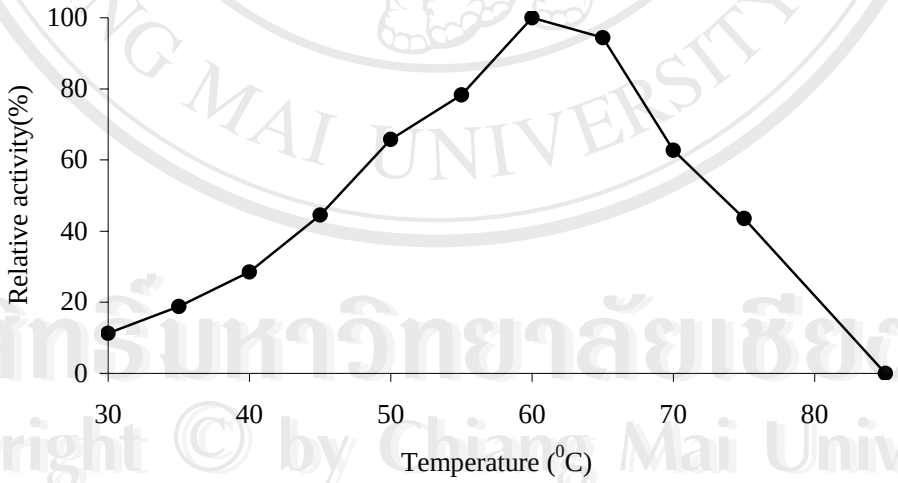

Figure 4.4 Effect of temperature on aminopeptidase activity

Table 4.5 Optimum temperature and thermostability of aminopeptidase from various bacteria

Sources	**Optimum temperature (°C)**	**Thermostability (°C)**	**References**
Lactobacillus sanfrancisco CB1	30-35	NR	Gobbetti *et al*., 1996
Pyrococcus horikoshii	>90	95	Ando *et al*., 1999
Lactobacillus casei subsp. *rhamnosus* S93	39	<45	Arora and Lee, 1994
Streptococcus thermophilus A	37	NR	Chavagnat *et al.*, 1999
Bacillus sp. N2	50	NR	Lee *et al*., 1998
Bacillus kaustophilus CCRC11223	65	NR	Lin *et al*., 2004
Bacillus stearothermophillus	60	NR	Kuo *et al*., 2003
Geobacillus. thermoleovorans 47b	60	90	This study

NR: No result

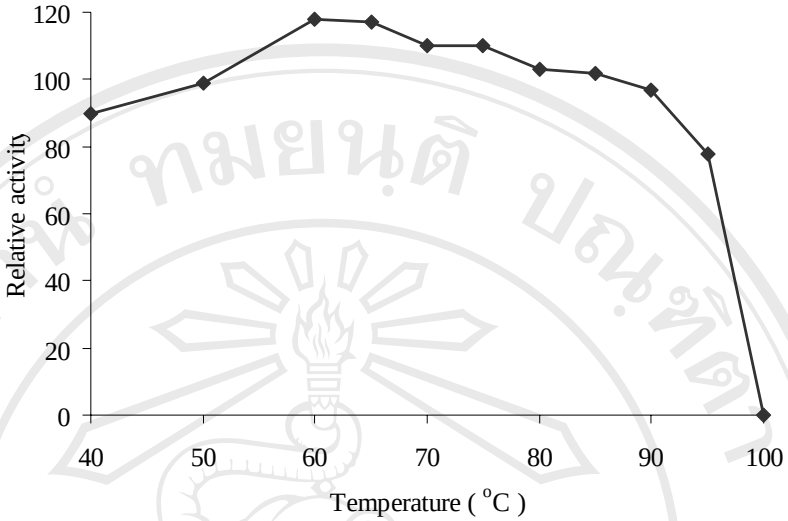

Figure 4.5 Effect of temperature on stability of aminopeptidase

Effect of chelating reagents on the enzyme activity

The effect of chelating reagents on the activity of the enzyme was showed in Table 4.6. The enzyme was strongly inhibited by metal chelating agents such as EDTA or o-phenanthroline and dipyridyl, indicating that aminopeptidase from *G. thermoleovorans* 47b required a metal ion for either stability or activity. This result showed that aminopeptidase from *G. thermoleovorans* 47bwas metalloenzyme.

Effect of cations on enzyme activity

The effect of cations on the enzyme activity was showed in Table 4.7. The aminopeptidase activity was strongly inhibited by Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} at 1 mM concentration and moderately inhibited by Fe^{2+} and Fe^{3+} at 0.1 mM. The aminopeptidase activity was slightly inhibited by Na^{+}, K^{+} and Mg^{2+}.

Table 4.6 Effect of chelating reagents on the aminopeptidase activity

Reagents	Concentration (mM)	Relative activity (%)
Control	-	100
EDTA	0.1	25
EDTA	1	14
EDTA	10	21
o-Phenanthroline	0.1	32
o-Phenanthroline	1	17
o-Phenanthroline	10	11
Dipyridyl	0.1	89
Dipyridyl	1	21
Dipyridyl	10	14

Effect of bivalent ions on enzyme activity

The metal ion requirement for the enzyme activity was tested by adding metal ions to the EDTA inactivated enzyme. After the enzyme was inactivated by dialyse in 10 mM KPB (pH 6.8) that contain 0.1 mM EDTA. The enzyme was dialyse against 10 mM KPB (pH 6.8) for 4 h twice, it inactivated by various metal ions. The aminopeptidase activity could be reactivated 219 % or 150 % by the addition of 0.2 mM Co^{2+} or 0.4 mM Co^{2+}, respectively (Table 4.8). However, incubation with Zn^{2+}, Mg^{2+} , Ca^{2+} ,Cu^{2+} and Mn^{2+} did not restored any enzyme activity. This result suggests that Co^{2+} is essential for the enzyme activity and confirmed that this enzyme was metalloenzyme.

Table 4.7 Effect of cations on enzyme activity

Metal ions	Concentration (mM)	Relative activity(%)
No addition	-	100
NaCl	1.0	97
KCl	1.0	95
$MnCl_2$	1.0	12
$MgCl_2$	1.0	84
$ZnCl_2$	1.0	17
$CuCl_2$	0.1	23
$CoCl_2$	1.0	17
$FeSO_4$	0.1	34
$FeCl_3$	0.1	40
$NiCl_2$	1.0	22

Table 4.8 Effect of bivalent ions on the aminopeptidase activity

Added ions (mM)	Relative activity (%)
Control	100.0
No addition	0.62
Zn^{2+} (0.2 mM)	24.0
Mg^{2+} (0.2 mM)	0.62
Ca^{2+} (0.2 mM)	8.00
Cu^{2+} (0.2 mM)	31.0
Mn^{2+} (0.2 mM)	4.00
Co^{2+} (0.2 mM)	219.0
Co^{2+} (0.4 mM)	150.0

Effect of chemical reagent on enzyme activity

To identify the particular type or class of aminopeptidase, the effect of various chemical reagents on the enzyme activity was examined (Table 4.9). The thiol group reducers DTT and β-mercaptoethanol, and the sulhydryl-directed reagent iodoactate completely inactivated the enzyme. Two other sulhydryl-direct reatgents, p-chloromercuricbenzoate (PCMB) and N-ethylmaleimide were less effective inhibitors. Phenylmethanesulphonyl fluoride (PMSF), and inhibitor of a number of serine proteases, and tosyl-L-phenylalanine chloromethyl ketone (TPCK), a specific inhibitors of chymotrypsin-like serine proteases, had no effect on enzyme activity. The aminopeptidase inhibitor bestatin strongly inhibited enzyme activity.

Table 4.9 Effect of chemical reagents on aminopeptidase activity

Chemical reagents	Concentration (mM)	Relative activity (%)
No addition	-	100
ρ-Chloromercuric benzoic acid (PCMB)	0.1	70
N-Ethylmaleimide	0.5	85
Dithiothreitol	1.0	0.5
2-Mercaptoethanol	1.0	6.8
Iodoacetate	1.0	7.1
Phenylmethylsulphonyl fluoride (PMSF)	1.0	116
Tosyl-L-phenylalanine chloromethyl ketone	1.0	112
Bestatin	0.5	6.4

Effect of sodium chloride on the aminopeptidase activity

As shown in Figure 4.6, the enzyme activities of crude and purified enzyme were measured in the presence of 0-20 and 0-14 % NaCl, respectively, when L-Leu-pNA was used as the substrate. The result showed that the enzyme activity was decreased against to NaCl concentration. Thongthai and Siriwongpairat (1978) found

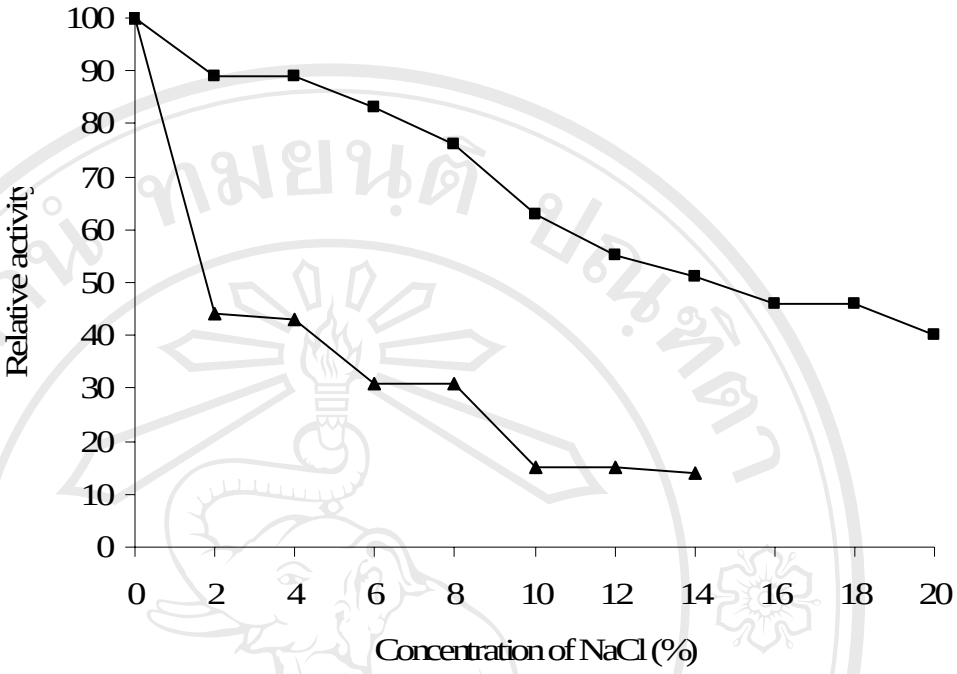

Figure 4.6 Effect of sodium chloride on the aminopeptidase activity in HEPES (pH 7.6) at 60 °C ; crude enzyme (■), purified enzyme (▲)

that sodium chloride generally to suppress protease activities, the higher the concentration the greater the degree of suppression.

Substrate specificity of purified aminopeptidase

The substrate specificity of the purified aminopeptidase from *G. thermoleovorans* 47b towards various p-nitroaniline derivatives was showed in Table 4.10. Among the substrates (p-nitroaniline derivatives) tested, the aminopeptidase was most active on Leu-pNA followed by Arg-pNA, Arg-Pro-pNA, Gly-Pro-pNA, but the enzyme showed very low activity towards Phe-pNA, Lys-pNA, Met-pNA, Gly-Phe-pNA, Gly-Arg-pNA, Ala- pNA, Gly-pNA, Pro-pNA, Ala-Ala-Ala-pNA and Val-Ala-pNA. Ala-Ala-Phe-pNA, Ala-Ala-Leu-pNA, Pyr-Phe-Leu-pNA, Suc-Ala-Ala-Ala-pNA, Suc-Ala-Pro-

Table 4.10 Substrate specificity of aminopeptidase from *G. thermoleovorans* 47b

Substrates	Relative activity (%)	
	Crude enzyme	Purified enzyme
Leu-pNA	100	100
Arg-pNA	56.3	82
Phe-pNA	9.2	7.7
Lys-pNA	5.0	4.1
Met-pNA	4.6	10.6
Ala-pNA	2.0	3.1
Gly-pNA	1.3	0
Pro-pNA	1.2	2
Arg-Pro-pNA	0	6.5
Gly-Pro-pNA	0	1.52
Gly-Phe-pNA	2.6	27.7
Gly-Arg-pNA	2.5	6.5
Val-Ala-pNA	0.7	3.0
Ala-Ala-Ala-pNA	1.0	4.8
Pyr[a)] -Phe-Leu-pNA	0	0
Suc [b)] -Ala-Pro-Ala-pNA	0	0
Suc-Ala-Ala-Ala-pNA	0	0
CBZ [c)] -Ala-Ala-Leu-pNA	0	0
CBZ-Gly-Gly-Leu-pNA	0	0

[a)] Pyr : Pyroglutamyl

[b)] Suc : Succinyl,

[c)] CBZ : benzyloxycarbonyl

-Ala-pNA and CBZ-Gly-Gly-Leu-pNA were inert. The results indicated that it was a leucine aminoeptidase. The purified aminopeptidase exhibited high substrate specificity was found to be narrow which was similar to the aminopeptidase from *Bacillus* sp. N2 (Lee *et al.*, 1998), *Bacillus stearothermophilus* (Kuo *et al.*, 2003). But the purified aminopeptidases from *Thermus aquatus* YT-1, (Minagawa, *et al.*, 1989) and *Lactobacillus plantarum* (Macedo *et al*., 2003) showed low substrate specificity.

Km and Vmax

The Michalis constant (Km) and Vmax) of purified aminopeptidase toward Leu-pNA were determined by various substrate concentrations. The linear Lineweaver–Burk plot showed in Figure 4.7. The Km and Vmax were 0.658 mM and 263.16 µmol/min/mg of protein, respectivley. The turnover rate (kcat) and catalytic efficiency (kcat/km) for Leu-p-NA was 10,179 S^{-1} and 15,470 $mM^{-1}S^{-1}$, respectively.

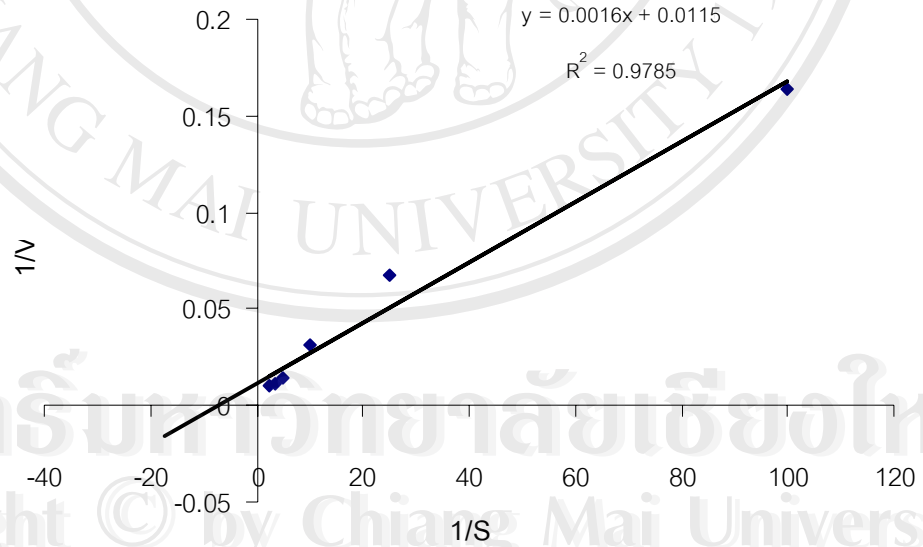

Figure 4.7 Lineweaver-Burk plot for hydrolysis of various concentration of L-Leu-pNA by aminopeptidase in 50 mM HEPES at 60 °C for 10 min.

Hydrolysis of bitter peptides by cell-free extracts and purified enzyme from *G. thermoleovorans* 47b

The mixture of bitter peptides solution was incubated with cell-free extracts of *G. thermoleovorans* 47b at 60 °C for 0, 1, 2, 3, 4, 8, and 16 h. The total amount of free amino acid concentrations were detected by TNBS method. As the incubation time elapsed, the amino acid concentrations of the enzyme reaction mixture increased in the amount of free amino acids being detected. The maximum amino acid concentration in the reaction mixture observed after incubation for 8 h, and slightly decreased at 16 h (Figure 4.8). Table 4.11 showed the profile of free amino acids released during the enzymatic hydrolysis and acid hydrolysis. Cell-free extracts of *G. thermoleovorans* 47b cleaved leucine in significant amount, suggesting that *G. thermoleovorans* 47b produces leucine aminopeptidase (LAP).

The incubation of mixture of bitter peptides solution with purified aminopeptidase was determined at 60 °C. The amount of free amino acid concentrations were detected after the incubated at various time for 0, 1, 2, 3, 4, 8, 16 and 24 h. It was found that the amino acid concentrations of the enzyme reaction mixture increased in the amount of free amino acids being detected. The maximum amino acid concentration in the reaction mixture was observed after incubation for 16 h, and the result showed slightly decrease at 24 h (Figure 4.8).

The incubation of bitter peptides solution with crude aminopeptidases showed the maximum amino acid concentration at 8 h, whereas incubation with purified aminopeptidase at 16 h that might be the cause of the other proteases hydrolysis in the reaction mixture.

The crude and purified aminopeptidase from *G. thermoleovorans* 47b was used to hydrolyze bitter peptides in comparison with leucine aminopeptidase from Porcine kidney and hydrochloric acid crude enzyme gave the highest level of leucine similar to Porcine kidney Leu-aminopeptidase, indicating that the bacterial crude enzyme might be Leu-aminopeptidase (Table 4.11). This result supported the substrate specificity data which was highly specific to Leu for both crude and purified enzymes. However, the hydrolysis result of the purified enzyme was not the same as the crude enzyme, probably due to the partial loss of enzyme activity during purification process and storage.

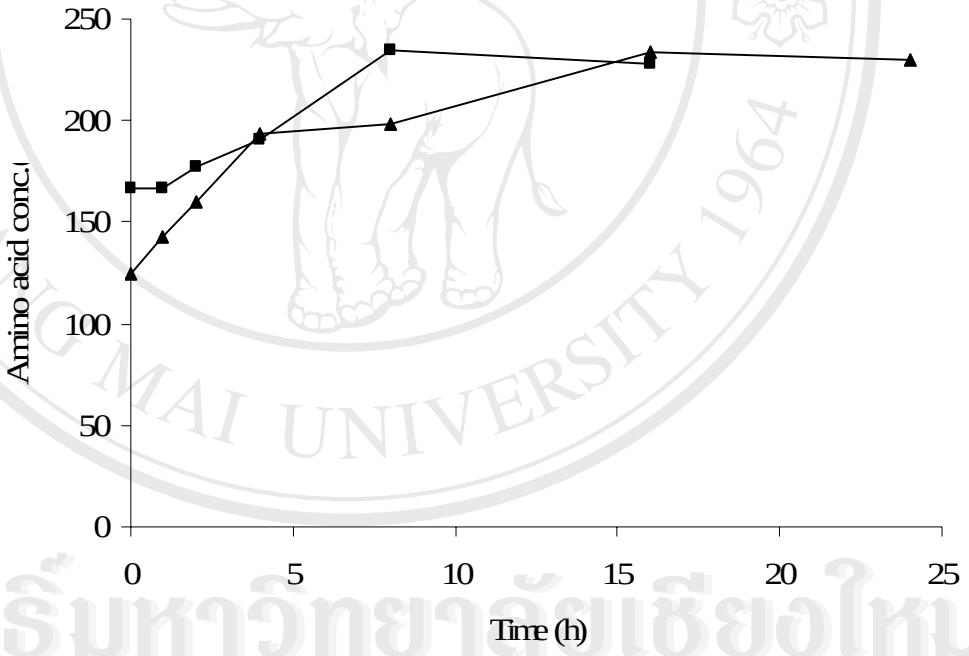

Figure 4.8 Time course of the hydrolysis of bitter peptides from *G. thermoleovorans* 47b, crude enzyme (■), purified enzyme (▲)

Table 4.11 Hydrolysis of bitter peptides by enzymatic or acid method

Amino acids	Cell-free extracts hydrolysis (mM)	Purified enzyme (mM)	Acid hydrolysis (mM)	Commercial Leucine aminopeptidase(mM)
Leu	7.3	0.3	20.6	18.1
Ile	0.4	0.4	11.8	7.6
Val	3.3	0.4	16.7	10.6
Ala	2.1	0.1	9.3	6.8
Met	1.0	0.3	2.5	3.9
Tyr	1.5	0	7.0	5.9
Phe	2.5	0	9.0	5.4
Trp	0.6	0.5	0	2.9
Ser	1.2	0.1	16.1	4.3
Thr	0.9	0	11.6	3.4
Cys	0	0.1	0	0.1
Pro	0	0.1	29.5	0
Gly	0.3	0.8	7.2	3.6
Asn	0.2	0	0	1.0
Gln	0.9	0	0	0
His	0	0.4	5.1	2.3
Lys	0.6	0	17.0	9.2
Arg	0.1	0.3	6.2	4.5
Asp	0.4	0.2	16.0	0.1
Glu	0.7	0	50.2	7.2