Comparative Study of Leucine Aminopeptidases from Marine Labyrinthulid and Thraustochytrid Strains

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Key words: labyrinthulid; thraustochytrid; leucine aminopeptidase; inhibitor

Abstract

Leucine aminopeptidases (LAPs) from marine labyrinthulid strain 00-Bat-05 and thraustochytrid strain HR-3 cells were partially purified and characterized by enzymological properties. The optimum temperature of LAPs from both strains was 37°C. The thermostability of 00-Bat-05 LAP was indicated by having 80% of maximum activity after heat treatment at 60°C for 10 min, while LAP activity of strain HR-3 was completely inactivated at 60°C. LAP acvtivities from both trains were high at near pH 8.0. Both LAPs were inactivated by 1,10-phenanthroline, *p*-chloromercuribenzoic acid (PCMB), bestatin and sodium dodecyl sulphate (SDS). Enzyme activity of LAP from strain 00-Bat-05 was stimulated by Co^{2+} and inhibited by Zn^{2+}, while that from HR-3 was inhibited by Co^{2+} and Zn^{2+}. LAP of 00-Bat-05 had a high specificity for L-leucine-*p*-nitroanilide but HR-3 enzyme showed relatively broad specificity for *p*-nitroanilide derivatives of L-amino acids.

Labyrinthulids (Family Labyrinthulaceae) and thraustochytrids (Family Thraustochytriaceae) are the main two groups of the order Labyrinthulales and play the peculiar roles in marine food web systems.[1-3] Labyrinthulids are slime mold-like protists and distributed widely in estuarine and near-shore marine habitats throughout the world. In mangrove brackish water environments, *Labyrinthula* spp. live on mangrove leaves, marine vascular plants and benthic algae, and also parasitizes fungal hyphae, which are decomposing plant matter. *Labyrinthula* spp. play an important role in mangrove ecosystem by fixing plant nutrients in a form of hyphae or cells of the organisms and converting them to a nutritious food for plant consuming animals.[4] The ectoplasmic networks of *Labyrinthula* spp. are capable of decomposing many different microbes including bacteria, yeast, diatoms, and fungal hyphae.[1] Thraustochytrids are a group of eukaryotic marine microorganisms, which can play dual roles in nature as bacterial feeders when in an ameboid form, and organic material degraders in their thallus form.[5] In mangrove environments, these marine protists are found to be associated with decaying mangrove leaves and decompose mangrove leaves by their degradative enzymes and also to be capable of breaking down several complex organic substrates.[6, 7]

In addition, these organisms also deserve more attention in aquaculture because they accumulate high concentrations of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) which are essential nutrients for many fish and crustaceans.[8-11] Thraustochytrids are used practically as a feed for zooplankton such as rotifers and *Artemia* because of improving the nutritional value of the food animals.[12]

In natural ecosystems, the recycling of nutrients, especially protein degradation is important to maintain all living things and aminopeptidases are found widely distributed among both prokaryotic and eukaryotic organisms, which catalyze the release of amino acid residues from the N-terminal end of proteins.[13] Leucine aminopeptidase (LAP, EC 3.4.11.1) activity in marine environments indicated an ecological role by bacteria in aquatic biogeochemical cycles.[14] LAP was partially purified from pig intestinal mucosa at first and found to hydrolyze a wide variety of peptides. The LAP activities from swine kidney and *Aspergillus sojae* was determined to be activated by divalent cations like Zn^{2+} and Co^{2+}.[15, 16]

The objective of this study was to characterize and compare the leucine aminopeptidase (LAP) from marine

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labyrinthulid and thraustochytrid isolates in order to assess nutritional process and degradation activity of these organisms in marine environments.

Materials and Methods

Labyrinthulid and thraustochytrid strains

Labyrinthulid strain 00-Bat-05 was isolated from mangrove leaves at the Batan Bay mangrove area, the Philippines, using a double layer medium containing live diatom cells.[17] Thraustochytrid strain HR-3 was provided by Dr. M. Hayashi, Faculty of Agriculture, Miyazaki University, Japan and which was isolated from costal area of Hiroshima Prefecture, Japan. Chromosomal DNA of the strains was extracted and 18S rDNAs were amplified by PCR and sequenced by the standard method. A phylogenetic tree inferred from 18S rDNA nucleotide sequences was constructed by the neighbor-joining method.[18]

Media and growth conditions

For the enzyme production, labyrinthulid strain 00-Bat-05 was grown at 25°C for 168 h in 1 *l* of the ES (Provasoli's enrichment seawater) liquid medium in a 2 *l* Erlenmeyer flask with containing bacterial extract-egg yolk agar medium (NSBEY agar) according to Wahid *et al.*[19] Thraustochytrid strain HR-3 was cultured at 25°C for 96 h in 1 *l* of the medium containing 30 g glucose, 3 g yeast extract, 5 g polypeptone and 750 *ml* of artificial seawater (ASW, Herbst's formula composed of NaCl 30.0 g, KCl 7.0 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g and $CaCl_2 \cdot 2H_2O$ 1.0 g per *l*, pH 7.6) in a flask on a reciprocal shaker (Taitec, NR-3, Japan) at 120 rpm.

LAP enzyme preparation from the test organisms

1. Labyrinthulid strain 00-Bat-05

After incubation at the same conditions as above, the cells of labyrinthulid strain 00-Bat-05 collected from 8 *l* of liquid culture were washed with 50 mM Tris-HCl buffer (pH 7.6) and then suspended in 40 *ml* of 50 mM Tris-HCl buffer. The suspended cells were disrupted by sonication (4280S, Kaijo Denki, Japan) for 5 min, the cell debris was removed by centrifugation (12,000 x g for 20 min) and the supernatant was filtrated by 0.45 μm membrane filter (Advantec, Japan) and flow-through solution was used as a cell-free extract. Solid ammonium sulfate was added to the cell-free extract solution to give 50% saturation. After 2 h, the precipitate was removed and the enzyme protein fraction was precipitated from the supernatant with ammonium sulfate (90% saturation) and collected by centrifugation. The precipitate was dissolved with 50 mM Tris buffer and dialyzed overnight against 50 mM Tris buffer. The dialyzed enzyme solution was loaded onto a Toyopearl DEAE-650M column (Tosoh, Japan) previously equilibrated with 50 mM Tris buffer and the proteinous materials were eluted with 50 mM Tris buffer and 500 mM NaCl using a linear gradient. The active fractions were pooled and concentrated by ultrafiltration with a YM-3 membrane (Amicon, USA). The enzyme solution was then applied to a Toyopearl HW-55F column (Tosoh, Japan) equilibrated with 50 mM Tris buffer containing 100 mM NaCl and eluted with the same buffer. The active enzyme fractions were combined, concentrated by ultrafiltration with a YM-3 membrane.

2. Thraustochytrid strain HR-3

After incubation at the same conditions as above, the cells of thraustochytrid strain HR-3 collected from 500 *ml* of culture broth were washed with 50 mM Tris-HCl buffer (pH 7.6) and then suspended in 50 *ml* of the same buffer. The suspended cells were disrupted by sonication and the cell debris was removed by centrifugation and the supernatant was filtrated by 0.45 μm membrane filter. Solid ammonium sulfate was added to the crude enzyme solution to give 60% saturation. After 4 h, the precipitate was collected by centrifugation, dissolved with 50 mM Tris buffer and dialyzed overnight against 50 mM Tris buffer. The dialyzed enzyme solution was loaded onto a Toyopearl Super Q-650M column (Tosoh, Japan) previously equilibrated with 50 mM Tris buffer and the proteinous materials were eluted with 50 mM Tris buffer and 500 mM NaCl using a linear gradient. The active fractions of LAP enzyme were pooled and concentrated by freeze drying. The freeze dried enzyme preparation was suspended in 50 mM Tris-HCl buffer and then applied to a Toyopearl HW-55F column (Tosoh, Japan) equilibrated with 50 mM Tris-HCl buffer containing 150 mM NaCl and the proteins were eluted with the same buffer. The

active enzyme fractions were combined and concentrated by freeze drying.

Enzyme assay

Aminopeptidase activity was spectrophotometrically assayed by using L-leucine-*p*-nitroanilide (Leu-*p*-NA) as substrate described by Chien *et al.*[16] The standard assay condition was as follows: 200 μl reaction mixture consisted of 20 μl of 10 mM L-leu-*p*-NA solution, 100 μl of 50 mM Tris-HCl buffer (pH 7.6) and 80 μl enzyme solution and incubated at 37°C for 2 h. In addition, *p*-nitroanilide (*p*-NA) derivatives binding a series of L-amino acids were used at final concentration of 1 mM to measure the relative activity for LAP enzymes. Absorbance at 405 nm was determined by micro titer plate reader, MPR-A4I (Tosoh, Japan). All data are expressed as average values of duplicate experiments.

Effect of reaction temperature

By using the standard reaction mixture, enzymatic activity was determined at different temperatures between 4 and 80°C for 2 h incubation.

Effect of heat treatment

Enzyme thermostability was determined after preincubation of the enzyme solution in 50 mM Tris-HCl buffer (pH 7.6) at various temperatures (4-80°C) for 10 min and then the solution was cooled on ice for 5 min followed by the standard assay condition to measure residual enzyme activity.

Effect of reaction pH

Enzyme preparations were preincubated in 50 mM various buffer at 30°C for 10 min. The optimum pH for enzyme reaction was examined under the standard assay condition using 50 mM buffer solutions with various pHs: sodium phosphate buffer (pH 6.0-7.0), Tris-HCl (pH 7.0-9.0), carbonate buffer (pH 9.0-11.0) and glycine buffer (pH 11.0-13.0).

Effect of inhibitors

The effect of *p*-chloromercuribenzoic acid (PCMB), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), 1, 10-phenanthroline, iodoacetamide (IAA), bestatin and sodium dodecyl sulfate (SDS) on LAP activity was determined after preincubation with the enzyme and the chemicals (final concentration of 1 mM) in 50 mM Tris-HCl buffer (pH 7.6) for 30 min at 30°C. The remaining activity was assayed under the standard condition.

Effect of metal ions

After preincubating the enzyme with various cations, chelating reagents or chemical reagents in 50 mM Tris-HCl buffer (pH 7.6) for 30 min at 30°C. The remaining activity was assayed under the standard assay condition.

Chemicals used

The synthetic chromogenic substrates, L-leucine-*p*-nitroanilide (Leu-*p*-NA), L-alanine-*p*-nitroanilide (Ala-*p*-NA) and L-glutamine-*p*-nitroanilide (Glu-*p*-NA) were obtained from Peptide Institute, INC. (Osaka, Japan) and L-proline-*p*-nitroanilide (Pro-*p*-NA), L-methionine-*p*-nitroanilide (Met-*p*-NA), L-arginine-*p*-nitroanilide (Arg-*p*-NA) were from Sigma Chemical Co. (St. Louis, Mo., USA). The protease inhibitors used in this study were purchased from Sigma and Nakarai Tesuque (Kyoto, Japan).

Results

Phylogenetic analysis of strains 00-Bat-05 and HR-3

Figures 1 A abd 1 B show cell morphology of labyrinthulid strain 00-Bat-05 and thraustochytrid strain HR-3, respectively. The individual cells of labyrinthulid 00-Bat-05 (Fig. 1A) were typically spindle form with averaged 2.5-5.0 μm wide by 8.0 -12.0 μm long. On the other hand, those of thraustochtrid HR-3 (Fig. 1B) were round form with 10.0-15.0 μm in diameter, which contained numerous granules in the cytoplasm. The 18S rDNA sequence of strain 00-Bat-05 (GenBank accession no. AB290459) showed 93% homology with that of *Labyrinthula* sp. AN-1565 (AB022105), while strain HR-3 had 99% homology with Thraustochytriidae sp. NIOS-1 haplotype NIOS1-D00-1 (AY705769). A phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 2.

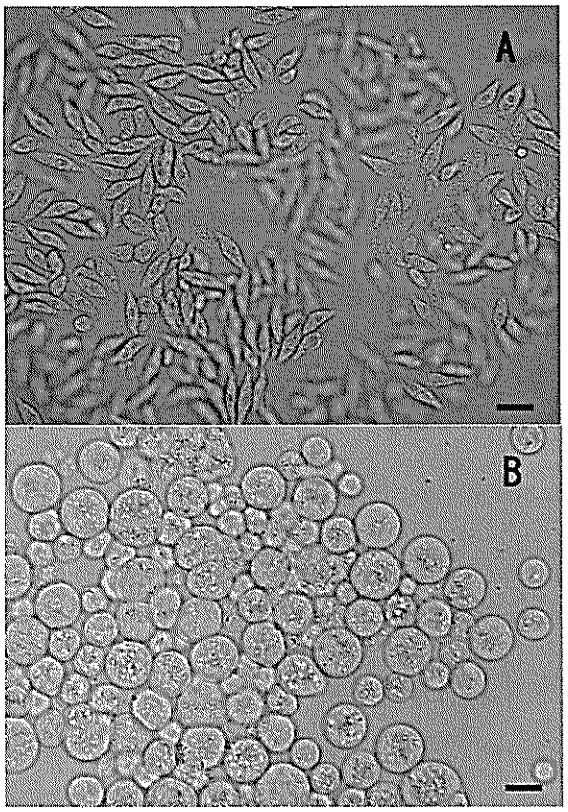

Fig. 1. Photograph of vegetative cells of labyrinthulid strain 00-Bat-05 (A) and thraustochytrid strain HR-3 (B). Scale bars indicate 10μm.

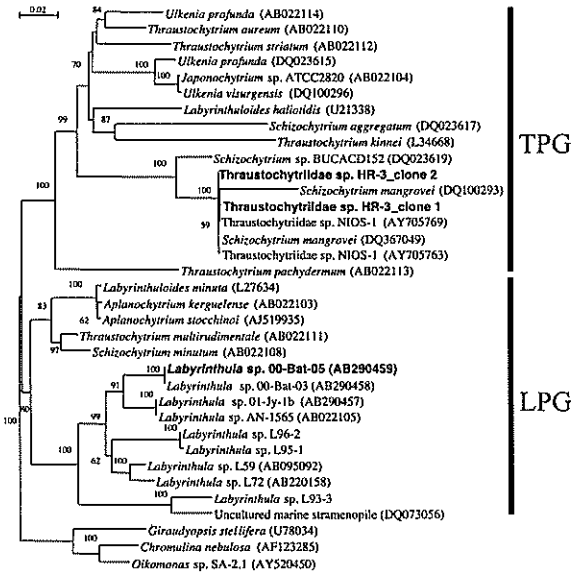

Fig. 2. Phylogenetic tree inferred from 18S rDNA of labyrinthulid sp. 00-Bat-05 and thraustochytrid sp. HR-3 by the neighbour-joining method. Bootstrap values greater than 50% are shown above the branches. A scale bar indicates genetic distance. LPG, labyrinthulid phylogenetic group; TPG, thraustochytrid phylogenetic group.

Enzyme preparation

Partially purified enzyme preparations were obtained after ion-exchange (Fig. 3) and gel filtration chromatography. One active fraction of LAP from each test strain was isolated although it still included several protein molecules as judged from polyacrylamide gel electrophoresis (PAGE). In this experiment, the partially purified enzymes were used for characterization of LAP activity.

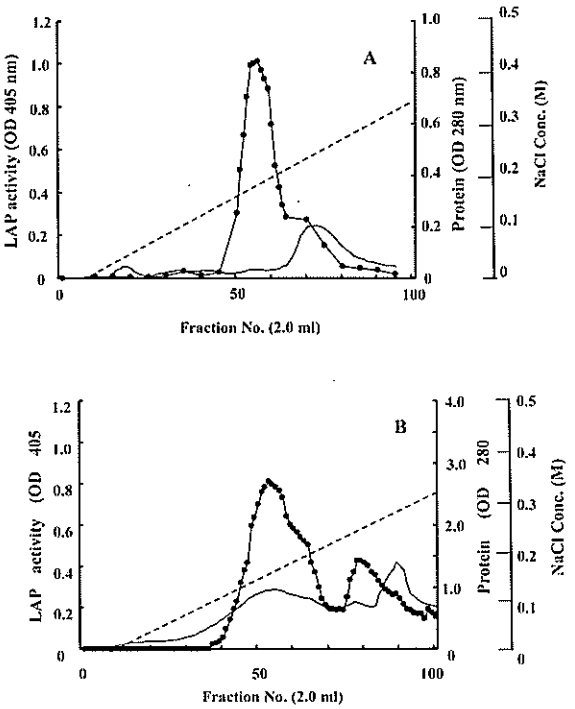

Fig. 3. Chromatographic profiles of LAP enzymes from test strains. A, Toyopearl DEAE-650M chromatogram of LAP from strain 00-Bat-05; B, Toyopearl Super Q-650M chromatogram of LAP from strain HR-3. ●: LAP activity (OD at 405 nm), —: protein concentration (OD at 280 nm), ---: NaCl concentration (M).

Characterization of LAP enzymes

1. Optimal temperature

LAP activities of both strains were at their maxima at 37°C as shown in Fig. 4. More than 50 % of maximum activity was found between 15 to 45°C in both strains. More than 80% activity of 00-Bat-05 LAP was remaining after heat treatment at 60°C for 10 min, while HR-3 LAP enzyme was almost inactivated with heat treatment at the same temperature (Fig. 5).

2. Optimal pH

As shown in Fig. 6, the optimum pH was 8.0 for 00-Bat-05 LAP and 7.0-8.0 for HR-3 LAP. However, the enzyme activity of 00-Bat-05 strain was found to be considerably low at pH 9.0.

3. Effect of inhibitors

Effect of various inhibitor substances on LAP activity is shown in Table 1. In strain 00-Bat-05, the LAP activity was strongly inhibited by bestatin, which is generally known to be an inhibitor of aminopeptidase (Table 1). Strong inhibition was also observed in the presence of EDTA, SDS, PCMB, DTT, and 1,10-phenanthroline. EDTA was known to be a metal-chelating agent, and PCMB to be a SH inhibitor. On the other hand, LAP activity from HR-3 strain was inhibited by bestatin, SDS, PCMB, and 1,10-phenanthroline.

4. Effect of metal ions

The effect of several metal ions on enzyme activity is shown in Table 2. Among the metal ions tested in LAP of strain 00-Bat-05, enzyme activity was strongly inhibited

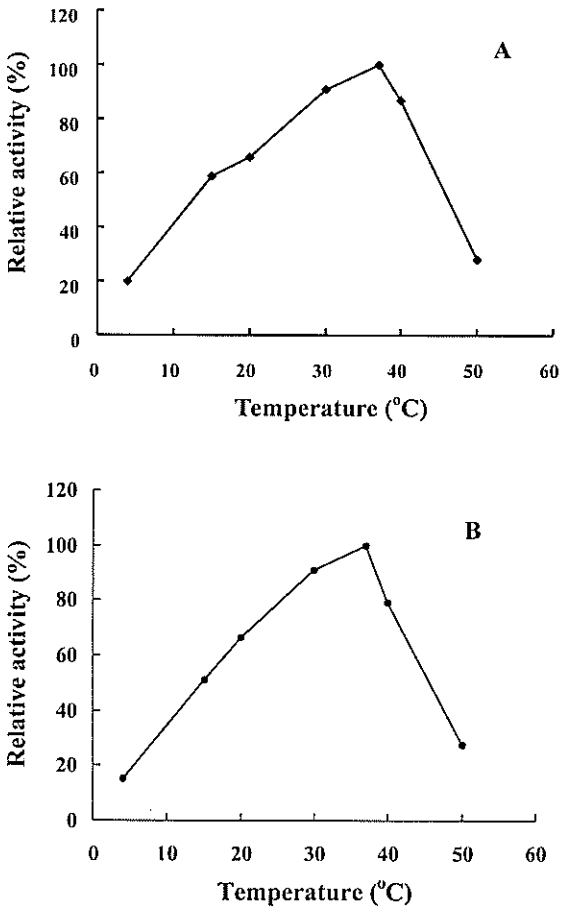

Fig. 4. Effect of reaction temperature on enzyme activity from strains 00-Bat-05 (A) and HR-3 (B). Enzyme activity was measured at various temperatures in 50 mM Tris-HCl buffer (pH 7.6). Activities are expressed relative to the maximum value.

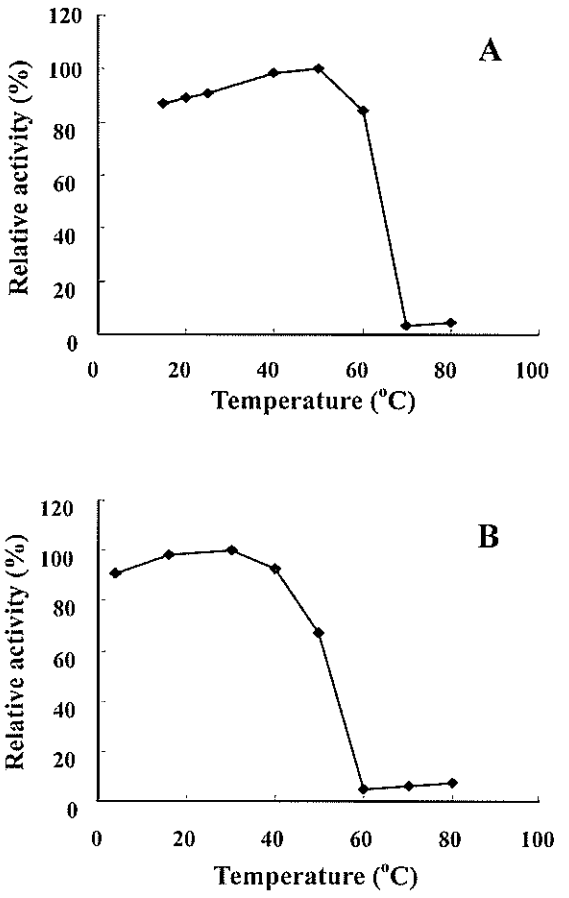

Fig. 5. Effect of heat treatment on enzyme stability of strains 00-Bat-05 (A) and HR-3 (B). Enzyme preparations were heated in 50 mM Tris-HCl buffer (pH 7.6) at various temperatures for 10 min

by Zn^{2+}, Hg^{2+} and Ni^{2+} and moderately inhibited by Mn^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+} and K^{+}. However, Co^{2+} strongly activated the enzyme activity from strain 00-Bat-05 . On the other hand, the LAP activity of strain HR-3 was strongly inhibited by Zn^{2+}, Hg^{2+} and Ni^{2+}, and slightly inhibited by Co^{2+}. Metal ions such as Na^{+}, K^{+}, and Mg^{2+} stimulated the LAP activity of strain HR-3.

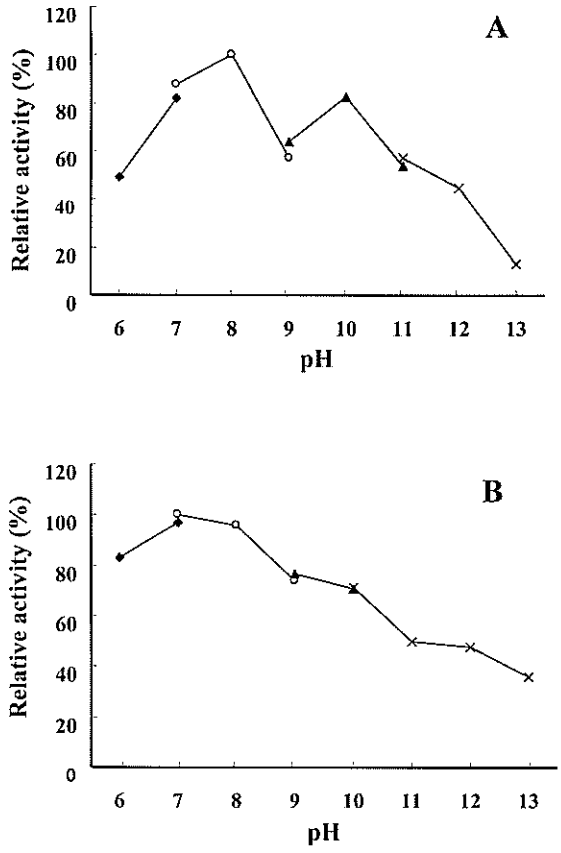

Fig. 6. Effect of pH on enzyme activity of strains 00-Bat-05 (A) and HR-3 (B). Enzyme preparations were preincubated in 50 mM various pH buffer at 30°C for 10 min. ◆: sodium phosphate buffer, ○: Tris-HCl buffer, ▲ : carbonate buffer, ×: glycine-NaOH buffer.

Table 1. Effect of chemical reagents on LAP activity from test strains

Chemical reagents*1	Relative activity (%)	
	00-Bat-05	HR-3
No addition	100.0	100.0
EDTA*2	40.2	88.0
PCMB*2	13.6	10.0
DTT*2	16.4	78.0
IAA*2	132.4	91.4
1,10-phenanthroline	12.8	16.0
PMSF*2	106.8	89.0
SDS*2	3.4	17.5
Bestatin	5.2	35.7

*1Each chemical reagent was added to a final concentrqaion of 1 mM in the reaction mixture.

*2EDTA:ethylendiaminetetraacetic acid, PCMB: *p*-chloromercuribenzoic acid, DTT: dithiothreitol, IAA: iodoacetamide, PMSF: phenylmethylsulfonyl fluoride, SDS: sodium dodecyl sulfate.

Table 2. Effect of metal ions on LAP activity from test strains

Metal ions[1]	Relative activity (%)	
	00-Bat-05	HR-3
No addition	100.0	100.0
NaCl	108.5	187.8
KCl	55.8	155.7
$MnCl_2$	76.0	104.3
$MgCl_2$	62.8	178.8
$ZnCl_2$	31.0	29.7
$CuSO_4$	52.7	100.0
$CaCl_2$	79.9	99.5
$HgCl_2$	30.2	23.1
$NiCl_2$	12.9	46.7
$CoSO_4$	298.6	75.0
$BaCO_3$	55.5	.87 3

[1] Each salt was added to a final concentraion of 1 mM in the reaction mixture.

5. Substrate specificity

The ability of the enzyme to catalyze the hydrolysis of various *p*-NA derivatives is shown in Table 3. In strain 00-Bat-05, Leu-*p*-NA was most efficiently hydrolyzed by LAP. Met-*p*-NA, Ala-*p*-NA and Arg-*p*-NA were also to be considerably hydrolyzed, while Pro-*p*-NA and Glu-*p*-NA were practically resistant to the action of the enzyme. In strain HR-3, Ala-*p*-NA was most efficiently hydrolyzed by the enzyme and Leu-*p*-NA, Met-*p*-NA and Arg-*p*-NA were good substrate for LAP. On the other hand, Pro-*p*-NA and Glu-*p*-NA were not optimal to the enzyme activity from stain HR-3.

Table 3. Hydrolytic activities of LAP from test strains on various substrates

Substrate[1]	Relative activity (%)	
	00-Bat-05	HR-3
Leu-*p*-NA	100.0	100.0
Ala-*p*-NA	21.0	347.0
Pro-*p*-NA	1.3	34.2
Met-*p*-NA	51.0	109.4
Arg-*p*-NA	20.1	125.0
Glu-*p*-NA	5.2	22.7

[1]All substrates were used at a final concentration of 1 mM under the standard assay conditions.

Discussion

Both labyrinthulids and thraustochytrids have a role in decomposing plant matters in coastal environments and their degrading enzymes are involved in this process.[1]

This study compared the enzymological properties of LAP enzymes from labyrinthulid and thraustochytrid strains isolated from marine environments.

The LAP enzyme of strain 00-Bat-05 had an optimal temperature of 37°C but 80% of its maximal activity remained even after heat treatment at 60°C for 10 min. On the other hand, the optimal temperature for the LAP activity of strain HR-3 was 37°C and the enzyme was inactivated completely with heat treatment at 60°C for 10 min. The optimum pH of LAP from strain 00-Bat-05 was 8.0 and high activity was still detectable at pH 10.0 and 11.0. However, the activity was relatively low under neutral conditions (pH 7.0). The HR-3 LAP had a broad pH range from 6.0 to 11.0 and the activity was still found about 50% at pH 11.0 and 12.0. These results indicated that both enzymes have optimal activity at alkaline region similar to other aminopeptidases.[16, 20]

The LAP activities from strains 00-Bat-05 and HR-3 were inhibited by PCMB, suggesting both LAP enzymes to be SH enzymes. The LAP activity from 00-Bat-05 was inhibited by 1,10-phenanthroline and EDTA and similar results were found in LAP from *Geobacillus thermoleovoran*.[21] Most of LAP enzymes from bacteria and fungi were reported to be inhibited by metal-chelating agents and to be largely restored by divalent cations such as Zn^{2+} and Co^{2+}.[16, 20] The LAP activity of strain 00-Bat-05 was inhibited by Zn^{2+} and stimulated by Co^{2+}, while the enzyme activity of strain HR-3 was slightly inhibited by Co^{2+} and strongly inhibited by Zn^{2+}. The enzyme of strain 00-Bat-05 was inhibited by K^+, but the enzyme of strain HR-3 was distinctively activated by Na^+ and K^+. These results indicate that effect of metal ions on LAP activity varies from organism to organism.

As substrates, Leu-*p*-NA was best for the LAP enzyme of strain 00-Bat-05 LAP, followed by Met-*p*-NA (51 %), Ala-*p*-NA (21 %) and Arg-*p*-NA (20 %) and negligible activity was detected for Glu-*p*-NA and Pro-*p*-NA. On the other hand, the enzyme of strain HR-3 showed high activity for Leu-*p*-NA (100 %), Ala-*p*-NA (347 %), Met-*p*-NA (109 %) and Arg-*p*-NA (125 %) and slight activity for Glu-*p*-NA and Pro-*p*-NA.

This is the first report on the isolation and enzymological properties of LAP enzymes from labyrinthulid and thraustochytrid strains. The information obtained in this study will help to reveal the ecological roles in degradation process of plant materials and microbial cells in marine environments and physiological response of these organisms as live food of aquaculture animals.

Acknowledgments

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