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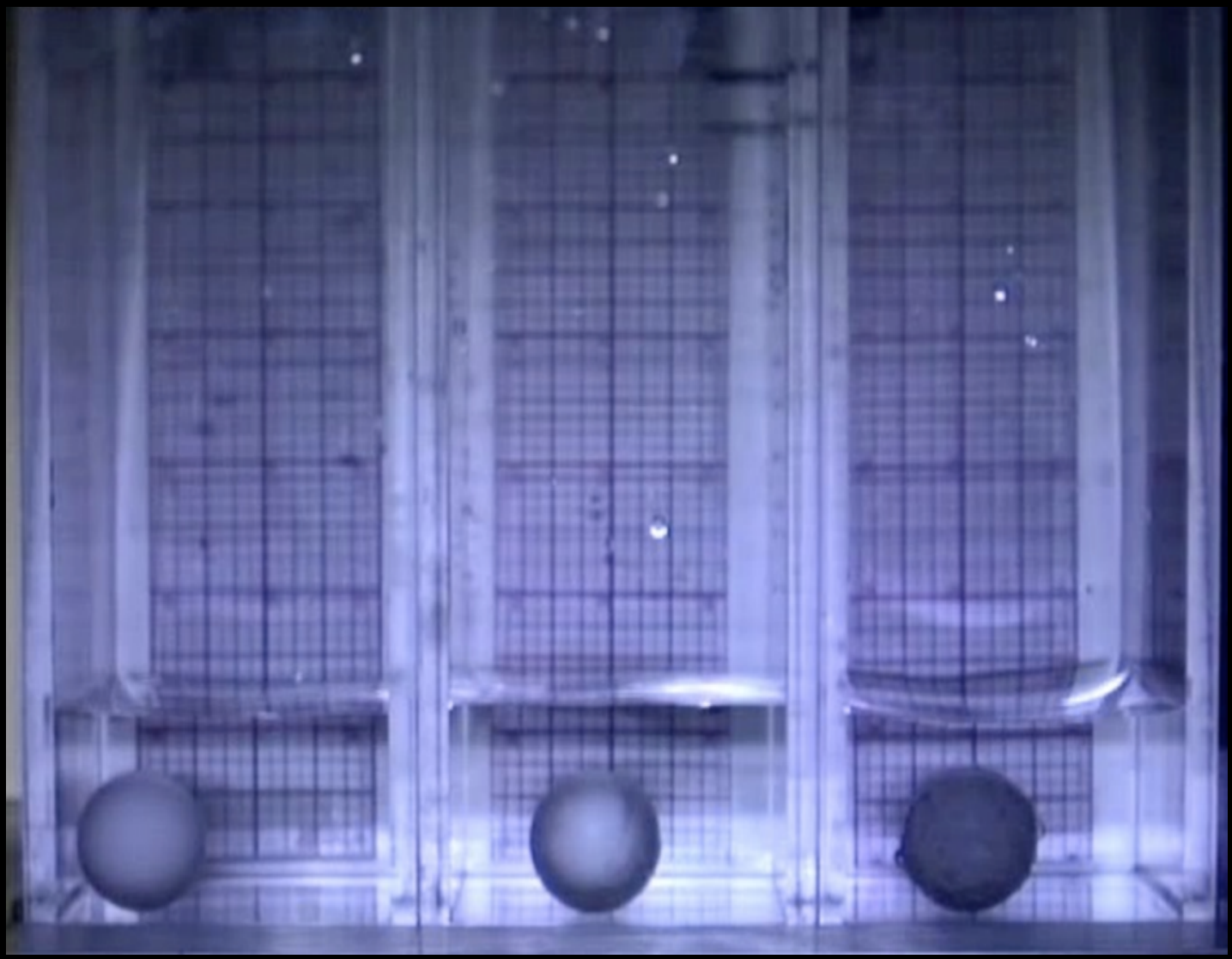

Expulsion Effect of Superhydrophic Materials

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Original Article

A Metalloaminopeptidase from *Flavobacterium breve:* Characterization and Molecular Cloning

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Abstract
Aminopeptidase from *Flavobacterium breve*, was purified by a three step FPLC column chromatography to homogeneity from the culture filtrate. The aminopeptidase gene was cloned by using TAIL-PCR technique. The gene encodes for a polypeptide composed of 497 amino acids with a theoretical molecular weight of 58 kDa. SDS-PAGE detection revealed that the protein is of 52 kDa. The native enzyme showed high affinity to Leu-pNA (k_m 0.0515 mM), and k_{cat} /k_m of 88.8 $s^{-1}mM^{-1}$. The enzyme had an optimum pH 7.5 and was stable from pH 6 to 9. The purified aminopeptidase was stable up to 60 ºC and the optimum temperature for the maximum activity was at 70 ºC. The amino acid sequence showed 47% identity to aminopeptidase of *Aeromonas caviae* (family M14), a Zn^{2+} dependent metallozyme.

Introduction
Aminopeptidases comprise a family of zinc metalloproteinases that catalyze the removal of N-terminal amino acid residues from various protein substrates. The endo-and exo- peptidases can easily find their niche in several branches of the food industry where free amino acids and short peptides, play positive roles in eliciting characteristic flavors of foods. Moreover, aminopeptidases are essential for protein maturation,[1] degradation of non-hormonal and hormonal peptides[2] and many diseases associated with impaired proteolytic function.[3-5] Aminopeptidases also eliminate bitterness in protein hydrolysates, by the hydrolysis of peptides containing hydrophobic amino acids responsible for the bitterness[6,7] and also accelerate ripening of cheeses.[8,9] Several types of cheeses when treated with aminopeptidases displayed excellent flavor with negligible accumulation of undesirable bitter peptides, [7,8] and other off flavors.[9]

The aminopeptidase from *Aeromonas proteolytica* has two zinc ions in its active site 3.5A apart, bridged by an aspartate and water molecule.[10] In case of Bovine lens leucine aminopeptidase, the N-terminal group of the substrate binds to one of the Zinc ions while the scissile carbonyl group binds to the other zinc ion.[11] Most of the reported metalloaminopeptidases are economically and industrially important. The search for new aminopeptidases is thus equally important to industrial application. The strains of *Flavobacterium* were capable of substantial growth on meat and dairy products and produce heat resistant proteolytic and lipolytic enzymes. Here, we explored the possibility to find aminopeptidase from the soil isolated bacterium *Flavobacterium breve* T2 strain. In the present investigation, aminopeptidase having specificity for large hydrophobic amino acid residues, was isolated and purified to homogeneity and characterized from *Flavobacterium breve T2*. The gene encoding the aminopeptidase was constructed from the deduced amino acid sequence. The homology with other aminopeptidases is verified.

Materials Methods
Cultivation of Flavobacterium breve
Flavobacterium breve T-2, (isolated from the soil in our Laboratory) was cultivated aerobically on a rotary incubator at a speed of 200 rpm at 30 ^{0}C for 42 h in Erlenmeyer flasks, in a medium composed of tryptone (2%), yeast extract (0.5%) and NaCl (0.5%) at pH 7.0. The cells were removed by centrifugation (8000 x g, 30 min, 4 ^{0}C) and the culture supernatant collected and immediately stored at 4 ^{0}C.

Purification of aminopeptidase
All the purification steps were carried out at room temperature or otherwise mentioned. After each step, fractions were assayed for aminopeptidase activity toward Leu-pNA, and active fractions were pooled. The supernatant was precipitated with solid ammonium sulfate to a final saturation of 90% at 4 ^{0}C. The precipitate was collected by centrifugation (10,000 *x g* for 20 min) and resuspended in 20 mM Tris-HCl buffer, pH 8.0 (buffer A) and desalted by dialysis. The solution was loaded onto a Q-sepharose FF column (Pharmacia, Uppsala, Sweden). The active fractions were pooled and dialysed overnight against buffer A at 4 ^{0}C and loaded onto a Butyl-Toyopearl 650 S (Tosoh Co., Tokyo) column equilibrated with bufferA. The absorbed proteins were eluted with a linear gradient of NaCl (0 to 1.0 M) of 20 mM Tris/HCl, pH 8.0 (bufferB). Fractions were separately dialysed against buffer A and loaded onto a Mono-Q (1mL) column and was eluted by linear gradient of buffer B. The active fractions were concentrated to 0.2 ml by micro-concentration (Microcon 3, Amicon Inc., Beverly, USA). The concentrated aminopeptidase solution was then subjected to gel filtration using a Superose column previously equilibrated with buffer B. The salt free active enzyme preparations were stored at –20 ^{0}C until further use. SDS polyacrylamide gel electrophoresis performed on 12.5% gels using the buffer system described by Laemmli.[12] Gels were stained for protein detection using Coomassie Brilliant Blue R25.

Amino acid sequencing

The protein was also transferred to nitrocellulose filter from the non-denaturing PAGE gel, as described by Sambrook et al (13), and was used for amino acid sequencing. The N-terminal amino acid sequence of the purified aminopeptidase was determined by the Edman-degradation method utilizing a G1000A protein sequencer (Hewlett Packard, Paolo Alto, CA). The resulting 45 N-terminal amino acids of the protein was subjected to protein homology search using the BLAST search.

Cloning of aminopeptidase gene

DNA manipulation was carried out using the standard protocols as described by Sambrook et al.[13] All products engineered by PCR were verified by DNA sequencing (ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit; ABI PRISM™ 310 Genetic Analyzer, PE Applied Biosystems). Plasmid DNA was prepared using Miniprep Kit (QIAGEN Ltd.) and digestion and ligation of DNA were performed as recommended by the manufacturer and were confirmed by agarose gel electrophoresis. QIAquick Gel Extraction Kit (QIAGEN Ltd., USA) was used for recovery of DNA fragments from agarose. The polymerase chain reaction (PCR) products were incorporated into pCR 2.1-TOPO plasmid (Invitrogen), and introduced into *E.coli* TOP10F' F'{lacIq Tn10 (TetR) mcrAD(mrr-hsdRMS mcrBC)F80lacZDM15 DLacX74 recA1 deoR araD139 D(Ara-leu)7697 galU galK rpsL (StrR) endA1 nupG } using the TOPO TA protocol (Invitrogen). Transformants were selectively grown on LB plates containing kanamycin (50 μg/ml).

A 3 stage PCR was designed consisting of a preliminary PCR stage followed by a thermal asymmetric interlacing (TAIL) PCR,[14,15] hemispecific PCR protocol, and concluding with a confirmatory proof reading PCR stage devised to determine the nucleotide sequence of the *Flavobacterium breve-AMP* gene. Alignment of the N-terminal peptide sequence of *Flavobacterium breve*-AMP with the peptide sequences of the bacterial leucyl aminopeptidase's of *Aeromonas caviae*-T64,[16] *Vibrio proteolytica*[17] and *Vibrio cholerae*[18] was initially utilized in determining highly conserved and homologous regions of the peptides. Oligonucleotide F1, 5'-TTGTAAAAGATTATATTACAACAAGT(A/C/G/T)AA(A/G)GA(A/G) GG/-3' (36-mer, degeneracy 16) encoded a sequence of 13 amino acids (IVKDYIT DVKEG) detected at the N-terminal. The oligonucleotides R1, 5-TA (T/C) GC(A/C/G/T)GC(T/A) GAAGAAG TAGGA TTACGT-3'(26-mer, degeneracy =16) and R2, 5'-GA(T/C)TA(T/C)AA(T/C)CCAAAGATTCATAC(T/A)ACA CAAGATAC-3' (35-mer, degeneracy 16) encoded 2 highly conserved internal amino acid sequences of the aligned Ac.AMP , Vc.AMP and Vp.AMP. Cycle conditions for the preliminary PCR were: 95 °C for 5 min, 95 °C for 30 s, 55 °C for 15 s and 72 °C for 30s (10.5 min on the last cycle) for 25 cycles. TAIL-PCR was utilized in further extending the reading of genomic DNA flanking the 750 base fragment of the *Flavobacterium breve-AMP* gene engineered during the preliminary PCR stage. Three forward and three reverse specific primers were selected from the 750 kb fragment and a single asymmetric degenerate (AD) primer, RPRDM1 (5'-AGWC(A,C, G,T)GAWG(A,C,G,T) ACWAAA-3';16-mer, degeneracy 128) was designed to complement the specific primers during each reaction of the 3 step TAIL-PCR protocol. TAIL-PCR was conducted using the thermal cycling conditions established by Liu and Whittier[14] following denaturation at 95 °C.

Confirmatory PCR was conducted using one set of sense (N-terminal, 5'-GTTGTGGAAAAGGCGAATATAC-3') and antisense (C-terminal, 5'-CTTAGGTTGGAT TGCTTATTTG-3') primers selected from the 2521 bases fragment engineered by TAIL PCR. The cycle conditions for the confirmatory PCR stage were as follows: 95 °C for 30 s, 55 °C for 15 s and 72 °C for 1.15 min (11.15 min for the last cycle) for 22 cycles. Chromosomal DNA of *Flavobacterium breve* cells were used as template in the preliminary, TAIL-PCR and confirmatory PCR. Thermocycling was carried out using a GeneAmp system 2400 (Perkin-Elmer) for the preliminary and confirmatory PCR stages and a GeneAmp sytem 9600 (Perkin-Elmer) for the TAIL-PCR stage.

Construction of aminopeptidase expression vector

An N-terminal (sense) primer 5'-ATCAAGACCA TGGTTCTGAACAAAATTTTAC-3' (31-mer) and C-terminal (antisense) primer 5'-GTCCTAAAGGAT CCGTCCGTTGTTTAAT GAATTAAG)-3' (32-mer), containing the restriction enzyme sites for *Nco*I and *Bam*HI respectively, were constructed for amplification by PCR of insert DNA (1532 base). Plasmid DNA was prepared from *E. coli* by using a Qiagen miniprep kit. DNA segments were separated by agarose gel electrophoresis, excised from the gel and purified by using a Qiagen gel-extraction kit. The gene was ligated (14 hrs, 16 °C) to the pET 28(a) expression vector (Qiagen) using the Toyobo ligation kit (Toyobo, Japan) to obtain the plamid containg the *Flavobacterium breve. AMP* gene.

Temperature- and pH-dependent Flavobacterium breve-AMP activity

The effect of temperature on *Flavobacterium breve*-AMP activity was measured in the range of 20 to 100 °C. The enzyme mixture was equilibrated for 5 min at the temperature tested before the addition of Leu-pNA substrate. The optimum pH for hydrolyzing Leu-pNA was determined in the pH range of 4 to 11 by using buffer consisting of 50 mM (each) Acetate buffer (4 to 5.8) N-morpholineethanesulfonic acid (MES) (pH5.2 to 7.2), morpholinepropanesulfonic acid (MOPS) (pH 7.0 to 8.0), (HEPES) (6.5 to 8.5), Tris-HCl (7 to 9), cyclohexylaminoethanesulfonic acid (CHES) (pH 8.2 to 10.1) and Cyclohexylaminopropane- sulfonic acid (CAPS) (9.4 to 11.0). The thermostability of the enzyme was demonstrated under the routine assay conditions after the enzyme solutions were treated for 30 min at the temperature tested. For measurement of pH stability, the activity was routinely assayed except at a final concentration of 250mM HEPES buffer (pH 7.5) after the enzyme solution had been pre-incubated for 30 min at the concentration of the above buffers.

1 ATGCTTCTGAACAAAATTTTACTACTTTCACTTTCTCTATTAAATGTTGGTCTCTTTGCA 60

M L L N K I L L L S L S L L N V G L F A 20

61 CAACATTCGCACAAACTACCAAAAGATACTCCCAAAAGTTACTTTTATGCAACGATGAAT 120

Q H S H K L P K D T P K S Y F Y A T M N 40

121 GCTGATCAGGCCGATAAATTAAAGGTTTTACATCCCAATGATGTTAAAATTTTAGCCATC 180

A D Q A D K L K V L H P N D V K I L A I 60

181 AACAAAAATGAAGCTGTAGTCAACATGAGCAATTATGCTGCTGCAGATTTACATCAATTT 240

N K N E A V V N M S N Y A A A D L H Q F 80

241 GTATTGTCGCATGGTCCAGGTTTTATACTTCATACGGATGAAAAAATCGCTAAAAATTAT 300

V L S H G P G F I L H T D E K I A K N Y 100

301 TTACAAAGACCACAAACCAAAACTTCAAGTGTTCTTGATTTTAACATTAGCGAAGATGAA 360

L Q R P Q T K T S S V L D F N I S E D E 120

361 ATTGTAAAAGATTACATCACACAAGTTAAAGAAGGAAATATACAAACGACCATACAAGCT 420

I V K D Y I T Q V K E G N I Q T T I Q A 140

421 TTAGAAGCAATTCATACACGATTTCATTTGTCAAACACTCGAAATGTCGGCATGGAATAC 480

L E A I H T R F H L S N T R N V G M E Y 160

481 ATCAAAAATTTGTGGCAATCCATTATTGATGAATCGGGAAGAGATGATTTGAAAGTCGAG 540

I K N L W Q S I I D E S G R D D L K V E 180

541 TTTTATACCCACAATAACACACCTCAATATTCAGTTATTTTCACGATCGAAGGCAACGAA 600

F Y T H N N T P Q Y S V I F T I E G N E 200

601 GAAGCAGATGAATACATCATTATTGGCGGTCATGCCGACAGTATTGTGAGTTCATCTTGG 660

E A D E Y I I I G G H A D S I V S S S W 220

661 GGCGGAAATTACGAATTGCGTTCACCTGGAGCTGATGATAATGCGAGTGGAATTGCCACC 720

G G N Y E L R S P G A D D N A S G I A T 240

721 GTTACAGAAGCGTTGAGAATATTGGTAGAAAATAGTTTTCGTCCCAAAAAAACCATTCAG 780

V T E A L R I L V E N S F R P K K T I Q 260

781 ATTATGGCCTATGCAGCCGAAGAAGTTGGATTGGTGGGATCTAATGAAATCGCTACAAAA 840

I M A Y A A E E V G L V G S N E I A T K 280

841 TATCGAAATCAAGGTATGGACGTCAAAGCATATGTACAATTTGACATGACCAATTATAAA 900

Y R N Q G M D V K A Y V Q F D M T N Y K 300

901 GGTTCTCCAAATGATGTTTACATCACTACAGATTCATACAATTCAAATGACCTAAATTTA 960

G S P N D V Y I T T D S Y N S N D L N L 320

961 TTTTTGGTCGAATTAATGGAACATTACAATGCTTCTGGAGATCATTCTTTTACTTATGGT 1020

F L V E L M E H Y N A S G D H S F T Y G 340

1021 TACACCATTTGTAACTATGGCTGTTCTGATCATGCATCGTGGGCAAACAAAGGTTTTCCT 1080

Y T I C N Y G C S D H A S W A N K G F P 360

1081 GCTGCTTTCCCTTTCGAGTCGAGTTTTAATGATAGTAATCCTAACATTCACACTAGTAAT 1140

A A F P F E S S F N D S N P N I H T S N 380

1141 GATACCTATTCAAAATCAAATGAAAGTAGTGCGCATGCCGCAAAATTTGCAAAATTAGCA 1200

D T Y S K S N E S S A H A A K F A K L A 400

1201 CTGCAATTTTTAGTAGAAGCGACTAAACCAACTGATGATTTAGGTCTAAATAACACCTCA 1260

L Q F L V E A T K P T D D L G L N N T S 420

1261 AAGAGCAATTCTAAAATAGTGGTGAATCAAAAAACATTAAACTATTTTTTAGAAAGCTCG 1320

K S N S K I V V N Q K T L N Y F L E S S 440

1321 ATGAATAATAACCGAGTGAAAATTATCAATCCAACAGGTCAAATTGTTTATCAAAACGAT 1380

M N N N R V K I I N P T G Q I V Y Q N D 460

1381 AAACTTTCATCAAATGGACAACTTAATTTAACACAATTAACCAATGGCATGTATATCGTT 1440

K L S S N G Q L N L T Q L T N G M Y I V 480

1441 GTTTTCGAATCCGATAAGGGTGAAAAATTCACTTCAAAATTCTTAATTCATTAAACAACAT 1500

V F E S D K G E K F T S K F L I H * 497

Fig. 1. Nucleotide sequence of the cDNA and the deduced amino acid sequence of aminopeptidase from *Flavobacterium breve*. Nucleotide residues are numbered from 5' to 3' starting with the first residue of the ATG codon encoding the putative initiating methionine. The deduced amino acid sequence is displayed below the nucleotide sequence as a single–letter code starting from methionine.

Measurement of aminopeptidase activity

Activity assays during cultivation and purification of the native *Flavobacterium breve*-AMP were conducted at 30 ^{0}C using 2mM Leu-pNA as the substrate.[19] One unit of aminopeptidase activity was defined as the amount of enzyme releasing 1 µmol/ min/ ml of pNA, under the standard assay conditions.

Determination of k_{cat} and k_m

Enzyme activity on p-nitroanilide derivatives of Leucine (Leu-pNA)) were measured at 30 ^{0}C under the standard conditions. The k_{cat} and k_m values of *Flavobacterium breve* -AMP on Leu-pNA were calculated, using different substrate concentrations. Calculations of k_{cat} and k_m values were carried out using the GraFit program (Erithacus Software, Staines, USA)

Determination of Protein content. Protein concentrations of *Flavobacterium breve*-AMP was determined spectrophotometrically at 280 nm and also measured by Bradford method with BSA as standard.

Results and Discussion

Purification of Flavobacterium breve-AMP from culture filtrate

So far there are no reports on aminopeptidase specific for Leucine from *Flavobacterium* species. *Flavobacterium breve* AMP was purified to homogeneity from the culture filtrate of *Flavobacterium breve* T-2, isolated in our laboratory, is of useful in dairy and other related food industries. The purification of aminopeptidase is summarized in Table 1. SDS-PAGE (Fig. 1) analysis of the gel-filtration fraction followed by activity staining showed the protein was homogenous and active, and had a molecular weight of 52 kDa. The amount of protein obtained from chromatographic separation was only 1.5 µg/mL. This enzyme hydrolyzed Leu-pNA much more than that of Met-pNA. The specificity for hydrolysis of Leu-pNA was 13.2 units/mg of protein, was higher than the values reported with porcine[20] and rat[21] aminopeptidases, reported early. The amino acid sequencing revealed the N-terminal sequence of 45 amino acids, when subjected to BLAST search showed 47% identity with

bacterial leucyl aminopeptidase of Family M14 and M28.

Characterization of Flavobacterium breve-AMP

The effects of temperature and pH on the activity of *Flavobacterium breve*-AMP were examined. The purified enzyme was stable for at least 30 min below 70 ºC, and its maximum activity was exhibited between 65 to 75 ºC (Fig. 3). The optimum pH for hydrolyzing Leu-pNA appeared to be 7.5 under the normal assay conditions, and no hydrolysis was detected below pH 4.0 (Fig. 4 a & b). The enzyme was stable over a broad pH range of 6.0 to 9.0. The relative rate of hydrolysis of Leu-pNA by the *Flavobacterium breve*-AMP was very high. It exhibited a very low (K_m 0.0515 mM), which shows the high affinity for the substrate and a strong catalytic activity K_{cat} /K_m of 88.8 $s^{-1}.mM^{-1}$. *Flavobacterium breve*-AMP showed less affinity to Met-pNA. Like other leucyl aminopeptidases reported, *Flavobacterium breve*-AMP also has the metal ion binding active site. The activity was completely inhibited by the addition of EDTA. The enzyme activity was recovered and enhanced by the addition Zn^{2+} (0.01mM). Thus this enzyme also can be included in the group of mettalozyme.

Table 1. Purification of native aminopeptidase of *Flavobacterium breve*

Purification Step	Volume (mL)	Total activity (Units)	Specific Activity (Units/mg)	Purification fold	Recovery (%)
Culture filtrate	4100	250	0.003	1	100
Amm.sulfate	150	126	0.05	17	50
Sepharose Q FF	100	3.7	0.25	83	1.5
Butyl Toyopearl	15	1.8	2.4	800	0.7
Mono Q	2.0	0.04	13.2	4400	0.016

Nucleotide sequence and deduced amino acid sequence

The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2. The ATG codon numbered as 1 must be the translation initiation site, since the nucleotide sequence around it corresponds to Kozak's rule.[22] The putative gene for aminopeptidase, isolated from *Flavobacterium breve* contained within the 2521bp length engineered by TAIL-PCR. The *Flavobacterium breve-AMP* gene was projected to consist of an open reading frame (ORF) of 1491 nucleotides.

A putative promoter region was located upstream of the initiation codon and consisted of 35 sequence of TTTACAA separated by 30 bp aminopeptidase from its complimentary 10 (TTCATT) sequence. A 28 bp palindromic sequence, a potential p-independent transcription terminator, was located downstream of the stop codon and can form a hairpin structure for mRNA with a delta G of –17.9 kCal/ mol.[23]

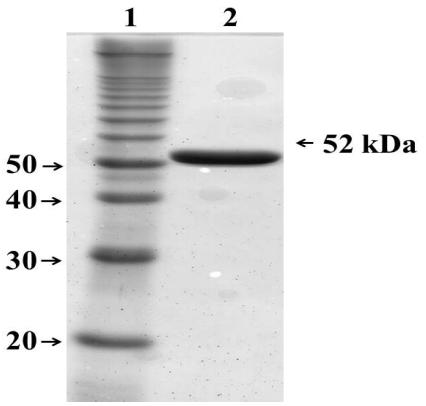

Fig. 2. SDS-PAGE of fractions obtained from Mono-Q purifications of the aminopeptidase.

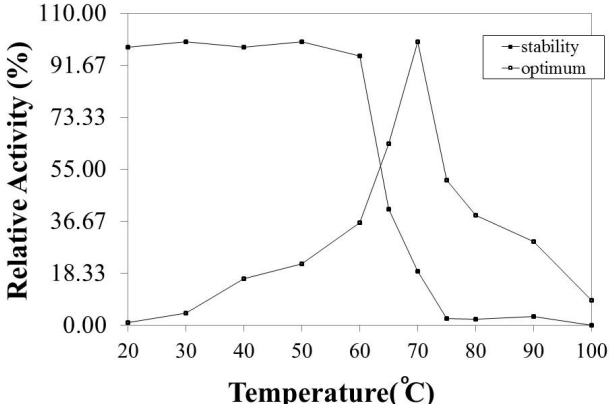

Fig. 3. Thermostability and optimum temperature of *Flavobacterium breve* aminopeptidase.

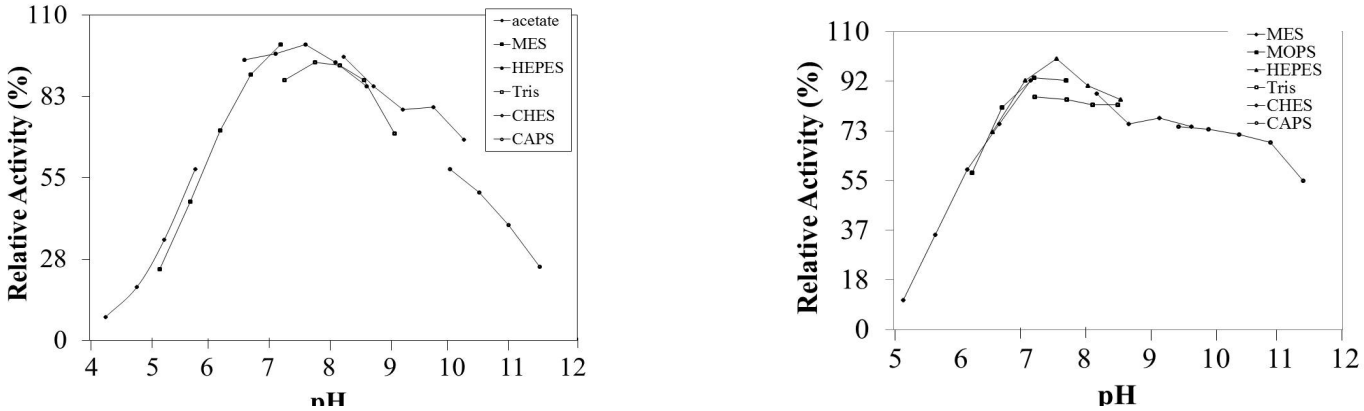

Fig. 4. (a) pH stability and (b) optimal pH of *Flavobacterium breve* aminopeptidase.

The ORF of 1491 base sequence was in agreement with the deduced amino acid sequence of 497 amino acids with a theoretical molecular weight of 58000 Da. A charged residue (Lys-5) was located within the first 5 amino acids and a core of at least 12 amino acid residues between Leu-2 to Phe-19 formed the hydrophobic motif of the signal peptide .[24] A helix breaking residue, Gly-17, occurs 4 amino acids from Ala-20, which is one possible terminal of the signal sequence given that 90% of known prokaryotic signal sequences end in either alanine or glycine. The rule[25] states that the residue at site 1 from the predicted junction must be small (Ala, Ser, Gly, Cys, Thr or Gln), and that at position 3 cannot be aromatics (Phe, His, Tyr and Trp), charged (Asp, Glu, Lys and Arg) or large and polar (Asn, Gln). In addition, the rule also stated that proline must be absent at positions 3 to 1. It is highly probable then that the signal sequence ends in Ala-20 as the junction Ala-20/ Glu-21 in aminopeptidase appears the most suited for cleavage.

The 45 amino acid N-terminal sequence of the purified aminopeptidase determined by Edman-degradation was located between Asp-113 and Met-158. In addition to the signal sequence the cloned nucleotide sequence appears to encode a pro-peptide of 92 amino acid residues. Long pro-peptides, which have been observed in several bacterial leucyl aminopeptidases have been proposed to act as regulators of enzyme activity. The aminopeptidase from *Aeromonas caviae* (Ac.AMP) possesses an N-terminal pro-peptide of 101 amino acids, while that of *Vibrio cholerae* (Vc.AMP) and *Vibrio proteolytica* (Vp.AMP) have been shown to be pre-proteins possessing pro-peptides at both N-terminal and at the distal C-terminal.[26,27] Alignment of the deduced *Flavobacterium breve*-AMP sequence with those of Vc.AMP and Vp.AMP suggests that the *Flavobacterium breve*-AMP is also synthesized as a pre-protein possessing both N and C terminal pro-peptide. The C-terminal pro-peptide of Vc.AMP (504 amino acids) and Vp.AMP (501 amino acids) begin at Leu-383 and Leu-386, respectively, extended to the end of the proteins and are of the same length (119 amino acids). The alignment of the deduced *Flavobacterium breve*-AMP peptide with Vc.AMP and Vp.AMp suggested that the C-terminal pro-peptide of the recombinant *Flavobacterium breve-AMP* is a shorter domain in *Flavobacterium breve-AMP* comprising 97 amino acids beginning at Leu-401. It is proposed that the recombinant *Flavobacterium breve* AMP may be synthesized as a preprotein comprising a signal sequence as well as long N and C-terminal propeptides.

In this study, aminopeptidase from *Flavobacterium breve* was isolated and partially characterized. The gene was constructed using the N-terminal amino acid sequence and by homology search. The constructed gene showed expected molecular weight when converted to amino acid sequence**.** Further studies are planned to clone the gene into an expression vector and characterize the recombinant protein. Investigations also planned to see any processing of the enzyme.

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Conflicts of Interest

There is no conflict of interest.

References

1. Moerschell RP, et al., Tsunawa S: The specificities of yeast methionine aminopeptidase and acetylation of amino-terminal methionine in-vivo. 1990, *J Biol Chem* 265:19638-43.
2. Botbol V, Scornik OA: Measurement of instant rates of protein degradation in the livers of intact mice by the accumulation of bestatin-induced peptides. 1991, *J Biol Chem* 266:2151-57.
3. Taylor A, et al., Jacques PF: Oxidation and aging: Impact on vision. 1993, G. Williams, ed, Proc Intl Conf on Antioxidants, vol. 8. Princeton, NJ: Princeton Scientific Press 349-71.
4. Dice JF: Cellular and molecular mechanisms of aging. 1993, *Phys Rev* 73:149-59.
5. Taylor A, Davies KJA: Protein oxidation and loss of protease activity may lead to cataract formation in the aged lens. 1987, *Free Radic Biol Med* 3:371-77.
6. Izawa N, et al., Hayashi K: Debittering of protein hydrolysates using *Aeromonas caviae* T-64. 1997, *J Agric Food Chem* 45:543-45.
7. Saha B, Hayashi K: Debittering Protein Hydrolyzates. 2001, *Biotechnology Advances* 19:355-70.
8. Hayashi K, Law BA: Accelerated ripening of cheddar cheese with the aminopeptidase of *Brevibacteria linens* and a commercial neutral proteinase. 1990, *J Dairy Res* 57:571-77.
9. Corsetti A, et al., Rossi J: Accelerated ripening of Pecorino Umbro cheese. 1998, *J Dairy Res* 65: 631-42.
10. Chevier B, et al., Tarnu, C: Crystal structure of Aeromonas proteolytica aminopeptidase: a prototypical member of the co-catalytic zinc enzyme family. 1994, *Structure* 2:283-291.
11. Strater N, Lipscomb WN: Two-metal ion mechanism of bovine lens leucine aminopeptidase: active site solvent structure and binding mode of L-leucinal, a gem-diolate transition state analogue, by X-ray

crystallography. 1995, *Biochemistry* 34:14792-800.
12. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. 1970, *Nature* 227:680-85.
13. Sambrook J, et al., Maniatis T: Molecular cloning: a laboratory manual. 1989, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
14. Liu YG, Whittier, RF: Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. 1995, *Genomics* 25:674-81.
15. Liu YG, et al., Whittier RF: Efficient isolation and maminopeptidase ping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. 1995, *Plant J* 8:457-63.
16. Izawa N, Hayashi K: Cloning and Nucleotide sequence of the aminopeptidase gene from *Aeromonas caviae* T-64. 1996, *J Ferment Bioeng* 82:544-48.
17. Heeke G, et al., Wagner FW: Cloning and nucleotide sequence of the *Vibrio proteolyticus* aminopeptidase gene. 1992, *Biochim Biophys Acta* 1131:337-40.
18. Toma C, and Honma Y: Cloning and genetic analysis of the *Vibrio cholerae* aminopeptidase gene. 1996, *Infect Immun* 64:4495-500.
19. Nirasawa S, et al., Hayashi K: Intramolecular chaperone and inhibitor activities of a propeptide from a bacterial zinc aminopeptidase. 1999, *Biochem J* 341:25-31.
20. Kawata S, et al., Makisumi S: Purification and some properties od porcine liver aminopeptidase B.1980, *J Biochemistry* (Tokyo) 88:1025-32 .
21. Hopsu VK, et al., Glenner GG: Characterisation of aminopeptidase B: substrate specificity and affector studies. 1996, *Arch Biochim Biophy*s 114:567-75.
22. Kozak M: An analysis of 5'-noncoding sequences for 699 vertebrate messenger RNAs. 1987, *Nucl Acids Res* 15:8125-48.
23. Tinco IJ, et al., Gralla J: Improved estimation of secondary structure in ribonucleic acids. 1973, *Nature New Biol* 246:40-41.
24. Watson MEE: Compilation of published signal sequences. 1984, *Nucl Acids Res* 12:5145-64.
25. von Heijne G: A new method for predicting signal sequence cleavage sites. 1986, *Nucl Acids Res* 14:4683-90.
26. Prescott JM, et al., Wilson KJ: *Aeromonas aminopeptidase*. 1971, *J Biol Chem* 246:1756-64.
27. Prescott JM, et al., Wilson KJ: Spectral and kinetic studies of metal-substituted Aeromonas aminopeptidase: nonidentical interacting metal-binding sites. 1985, *Biochemistry* 24:5350-56.