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Characterization of two M17 family members in *Escherichia coli*, Peptidase A and Peptidase B

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ABSTRACT

Escherichia coli encodes two aminopeptidases belonging to the M17 family: Peptidase A (PepA) and Peptidase B (PepB). To gain insights into their substrate specificities, PepA or PepB were overexpressed in Δ*pepN*, which shows greatly reduced activity against the majority of amino acid substrates. Overexpression of PepA or PepB increases catalytic activity of several aminopeptidase substrates and partially rescues growth of Δ*pepN* during nutritional downshift and high temperature stress. Purified PepA and PepB display broad substrate specificity and Leu, Lys, Met and Gly are preferred substrates. However, distinct differences are observed between these two paralogs: PepA is more stable at high temperature whereas PepB displays broader substrate specificity as it cleaves Asp and insulin B chain peptide. Importantly, this strategy, i.e. overexpression of peptidases in Δ*pepN* and screening a panel of substrates for cleavage, can be used to rapidly identify peptidases with novel substrate specificities encoded in genomes of different organisms.

1. Introduction

Intracellular proteolysis is responsible for the efficient turnover of cellular proteins and removing damaged or aged proteins. In addition, the cytosolic protein degradation pathway regulates several cellular processes [1]. One of the key players during intracellular protein degradation are aminopeptidases, several of which require metal as cofactor. Metallopeptidases are classified based on motifs and other features (www.merops.sanger.ac.uk) and the majority contains the HEXXH motif. The M1 family of peptidases contains GAMEN and HEXXH(X)$_{18}$E motifs which are important in catalysis and binding to Zn^{2+}. The remaining family members contain other motifs, e.g. HXXE which is typical of the M14 family, or conserved residues, e.g. Lys, Asp and Glu which are typical of the M17 family peptidases [2].

Initially, aminopeptidases were named based on the substrate used in an activity assay. This resulted in aminopeptidases being given names that were not truly reflective of their substrate specificities, for e.g., leucine aminopeptidases (LAP's). In general, LAPs belong to two peptidase families, M1 or M17, and their representatives are widely distributed and play diverse roles [3]. Mammalian M1 family members are important in several physiological processes: Aminopeptidase A regulates blood pressure [4] and angiogenesis [5]; LTA$_4$H generates inflammatory mediators [6]; ERAP1 trims MHC class I binding peptides in the endoplasmic reticulum [7], etc. PepN is the sole M1 family member in *Escherichia coli* and is responsible for majority of the aminopeptidase activity in total cell extracts [8]. M1 family members are also involved in protein degradation [9], stress responses [8,10–12] and have been characterized in pathogens such as *Salmonella typhimurium* [13] and *Plasmodium falciparum* [14].

A mammalian LAP belonging to the M17 family is IFNγ inducible and involved in N-terminal trimming of peptides generated by 20S proteasomes [15]. In addition, LAP, along with other peptidases, is important in degradation of crystalline protein deposits that accumulate in the eye after oxidative stress and/or aging [16]. A M17 LAP ortholog in tomato is induced upon wounding and modulates plant defense against pathogens [17]. *Plasmodium falciparum* encoded M17 LAP plays an important role in breakdown of hemoglobin in infected red blood cells [18]. *Escherichia coli* encodes two M17 family members: PepA and PepB. Interestingly, *E. coli* PepA binds DNA and is essential for Xer site specific recombination which is involved in stable inheritance of ColE1 plasmids [19]. The crystal structure of *E. coli* PepA reveals the existence of a DNA binding path, which mostly involves the N-terminal domain and certain extreme C-terminal residues [20]; however, the DNA-related activities and peptidase activity of PepA are independent of each other [21]. PepA orthologs in *Pseudomonas aeruginosa* [22] and *Vibrio cholerae* [23] act as negative regulators of virulence-associated genes. Inactivation of a *pepA* ortholog in *P. aeruginosa* correlates with the mucoid phenotype and increase in *algD* transcription. These studies clearly demonstrate a great amount of interest in studying the cellular roles of M17 family members in diverse organisms.

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Individual peptidases in *E. coli* and *S. typhimurium*, with the exception of methionine aminopeptidase [24], are not essential for growth due to the presence of redundant peptidases. However, there is evidence for roles of multiple peptidases during growth in minimal media [25] and degradation of selected substrates, e.g. cysteinylglycine [26] and microcin C [27]. *Escherichia coli* aminopeptidases, PepA, PepB and PepN, have overlapping substrate specificity and mutants of these peptidases exhibit some differences in peptide utilization compared to parent strains [28]. Further studies on the roles of individual peptidases in contributing to accumulation of trichloroacetic acid soluble peptides during growth in minimal medium revealed the following hierarchy: PepA > PepN > PepB and PepD [25]. However, these studies were done qualitatively and there is no quantitative and comparative study on the substrate specificity of PepA and PepB. As these two enzymes belong to the M17 family and are 26% identical and 17% similar, we characterized these enzymes using multiple approaches. Due to the major contribution of PepN to the aminopeptidase activities present in total cell extracts [8], PepA and PepB were overexpressed in Δ*pepN* and purified. As shown in this study, this strategy may be extended to other peptidases to rapidly identify novel substrate specificities.

2. Materials and methods

2.1. Strains and growth conditions

The *E. coli* strains used are listed in Supplementary Table 1. Strains were grown in LB (Himedia Labs, India) containing ampicillin (100 μg/ml) (Himedia) at 37 °C and 160 rpm.

2.2. Cloning of pepA and pepB

Genomic DNA extracted from *E. coli* K12 MG1655 was used to amplify *pepA* and *pepB* using *Pfu* polymerase (New England Biolabs, USA). The primers used for PCR amplification along with restriction enzyme sites are listed in Supplementary Table 2. The arabinose inducible vector pBAD24 and PCR amplified *pepA* and *pepB* were digested with NcoI and PstI (in case of *pepA*) and EcoRI and SmaI (in case of *pepB*). The products were gel eluted, ligated and transformed in competent DH5αΔ*pepN* cells. Plasmid DNA was isolated from transformed colonies and restriction digestion was performed to confirm the clones.

2.3. Preparation of total cell extracts

Different strains were grown for ~8 h at 37 °C in LB media with 400 μg/ml L-arabinose (Himedia). Bacteria were washed, sonicated and centrifuged at 100,000*g* for 1 h at 4 °C to obtain total cell extracts and 20 μg protein was used for enzyme assays.

2.4. Purification of PepA, PepB and PepN

Overnight grown cultures of *E. coli* transformed with pBAD24-*pepA*, pBAD24*pepB* and pBAD24*pepN* were grown for ~10 h at 37 °C with 400 μg/ml L-arabinose. To purify PepA, total cell extracts were obtained, subjected to heat denaturation at 60 °C for 30 min followed by centrifugation at 100,000*g* for 1 h. Proteins in the supernatant were precipitated using 50% $(NH_4)_2SO_4$, centrifuged at 100,000*g* for 1 h and the pellet was resuspended in 10 mM Tris–HCl, pH 8.0 followed by dialysis. The dialyzed proteins were loaded on DEAE-cellulose (Sigma, St. Louis, USA) and bound proteins were eluted with a 100–350 mM NaCl gradient in 10 mM Tris–HCl, pH 8.0.

To purify PepB, total cell lysates were precipitated using 50% $(NH_4)_2SO_4$, centrifuged, and the pellet was resuspended in 10 mM Tris–HCl, pH 8.0. After dialysis in 100 mM NaCl, the proteins were loaded on Q-Sepharose (Sigma), subsequently washed with 200 mM NaCl before eluting it with 200–350 mM NaCl. PepN was purified using a series of chromatography columns, e.g. DEAE-cellulose, Q-Sepharose and Butyl toyopearl (Sigma), as previously reported [8]. Further, Sephacryl-200 (Pharmacia) FPLC gel filtration was performed to obtain apparently homogenous purified proteins. Under native conditions, PepN migrates as a monomer of ~90 kDa whereas PepA and PepB are hexamers with molecular weights of ~340 and ~275 kDa, respectively. Enzyme purity was checked by SDS–PAGE with silver nitrate staining (Supplementary Fig. 1A).

2.5. Spectroscopic studies

Purified enzymes (~50 μg protein) in 400 μl of 20 mM phosphate buffer pH 8.0 were used and ellipticity was monitored from 200 to 250 nm in a 0.2-cm path length cuvette with a bandwidth of 1 nm and response time of 2 s at 20 °C. The molar ellipticity and thermal denaturation temperature (T_m) was calculated as previously reported [12].

2.6. Enzyme assays

Total cell extracts of different strains or purified enzymes (10 ng protein for aminopeptidase substrates and 1 μg protein for endopeptidase substrates) were assayed for hydrolysis of aminopeptidase (0.5 mM) and endopeptidase (0.5 mM) substrates, in 20 mM phosphate buffer, pH 8.0, at 37 °C for 1 h. All substrates used were conjugated to AMC (Bachem AG, Switzerland) with the sole exception of Lys which was linked to *p*NA (Sigma). The enzyme assays and kinetic parameters were performed as previously reported [11]. To study the cleavage of natural substrates, different amounts of purified PepA, PepB and PepN were incubated with 100 μg of either oxidized insulin B chain or casein (Sigma) in 20 mM phosphate buffer, pH 8.0 for 4 h. The release of newly released amino groups was assayed as previously reported [11].

2.7. Inhibitor analysis

The purified proteins were incubated with different inhibitors for 15 min followed by addition of 0.5 mM of L-Leu-AMC, at 37 °C for 1 h and AMC released was measured. The inhibitors used were: 5 μM pepstatin (aspartate protease); 5 mM EDTA; 5 mM EGTA; 4 mM 1,10-phenanthroline (metalloprotease); 1 mM DTT (cysteine protease) and 280 μM bestatin (aminopeptidase).

2.8. Growth analysis

Preinoculum (0.2%) of different strains were grown overnight at 37 °C and transferred to either LB medium or M9 minimal [14]. Bacterial strains were initially grown at 37 °C for 3.5 h and then shifted to 42 °C. Cultures were aliquoted at different time intervals and dilutions were plated on LB agar plates for CFU analysis.

3. Results

3.1. Substrate hydrolysis profile of PepA and PepB in total cell extracts of ΔpepN

In order to characterize the two M17 family members in *E. coli*, *pepA* and *pepB* were cloned in pBAD24, using the strategy shown in Fig. 1A. Following confirmation of the clones, the hydrolysis of a

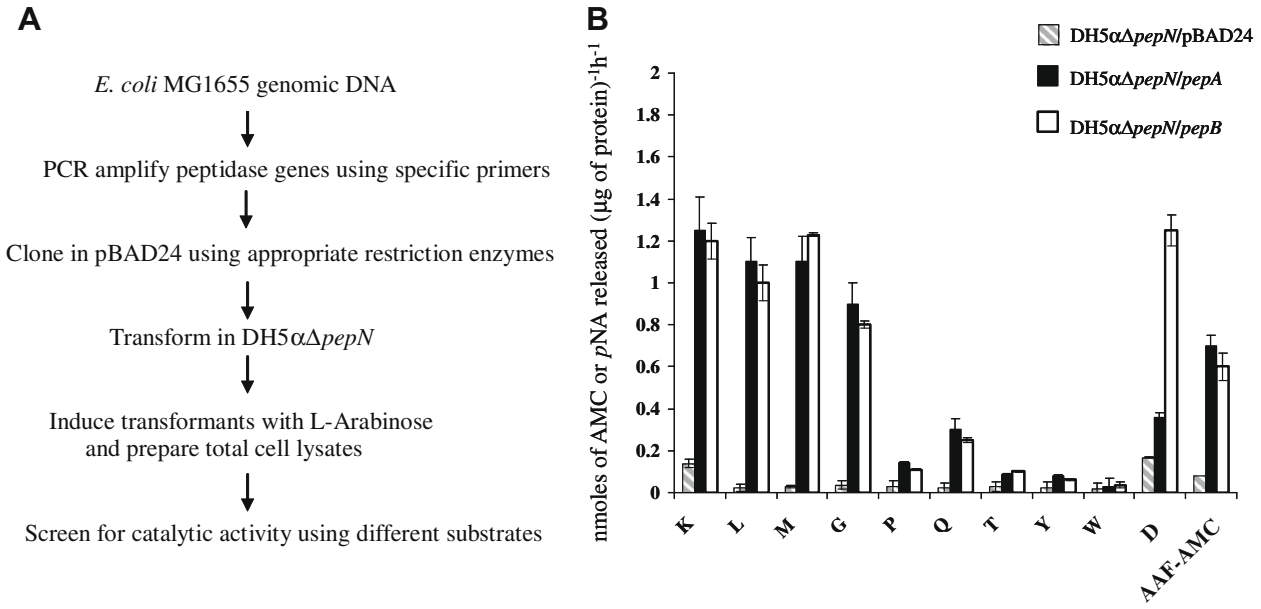

Fig. 1. PepA or PepB overexpression in Δ*pepN* increases catalytic activity for selected aminopeptidase substrates. The cloning strategy of *pepA* and *pepB* in Δ*pepN* and screening for activity is shown (A). Total cell lysates (20 μg) of indicated strains were incubated with different substrates (0.5 mM) for 1 h at 37 °C and the released AMC or *p*Na was measured. The specific activity is means ± SE representative of four independent experiments.

panel of 11 aminopeptidase substrates in total cell extracts of Δ*pepN* was studied. As shown in Supplementary Fig. 2, Δ*pepN* shows greatly reduced ability to cleave these substrates and overexpression of PepN greatly increases aminopeptidase activities [8]. Upon overexpression of PepA and PepB, the cleavage of few substrates, e.g. Lys, Leu, Met, Gly and AAF-AMC was increased by ~8–10-fold (Fig. 1B). Interestingly, only PepB showed significant cleavage of Asp. These results demonstrate that overexpression of PepA and PepB increased aminopeptidase activities in Δ*pepN*.

3.2. Effects of overexpression of PepA or PepB in ΔpepN during stress

PepN modulates the growth of *S. typhimurium* and *E. coli* during nutritional downshift and high temperature stress [11,12]. To study whether the increased peptidase activities in Δ*pepN* upon overexpression of PepA or PepB plays a role, indicated strains were grown at 37 °C for 3.5 h and then transferred to 42 °C in LB or minimal media (Fig. 2). No difference in growth among the different strains was observed in LB. However, Δ*pepN* showed reduced growth, as compared to WT, after transfer to minimal medium at 42 °C and PepN overexpression rescued this effect. Notably, overexpression of PepA or PepB partially rescued the growth of Δ*pepN* in minimal medium. Thus, the increased aminopeptidase activities upon overexpression of PepA and PepB partially improved the growth of Δ*pepN* during nutrition deprivation and high temperature stress.

3.3. Substrate specificities of PepA, PepB and PepN

To study the substrate preferences of PepA and PepB and compare them with PepN, these enzymes were purified to apparent homogeneity (Supplementary Fig. 1A). The cleavage pattern of PepN was Lys > Leu/Gly/Met > Pro/Thr/Tyr/Gln > Trp (Fig. 3A). However, the cleavage profile of PepA and PepB was distinct: Lys/Leu/Met/Gly > Pro/Tyr > Thr/Gln/Trp. Interestingly, PepB was the only enzyme that cleaved Asp. PepN cleaved the tripeptide sub-

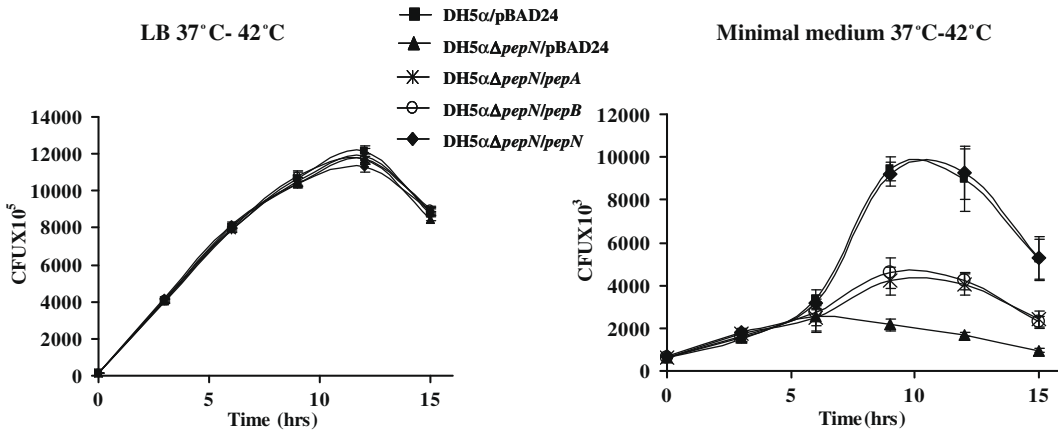

Fig. 2. Overexpression of PepA or PepB partially rescues the growth of Δ*pepN* during nutritional downshift and high temperature stress. Strains were grown overnight in LB and transferred either to LB or M9 minimal medium. Growth analysis was performed by growing cultures at 37 °C for 3.5 h followed by 42 °C. At indicated time points, different dilutions of the cultures were plated for CFU analysis. Data is shown as means ± SE of four independent experiments.

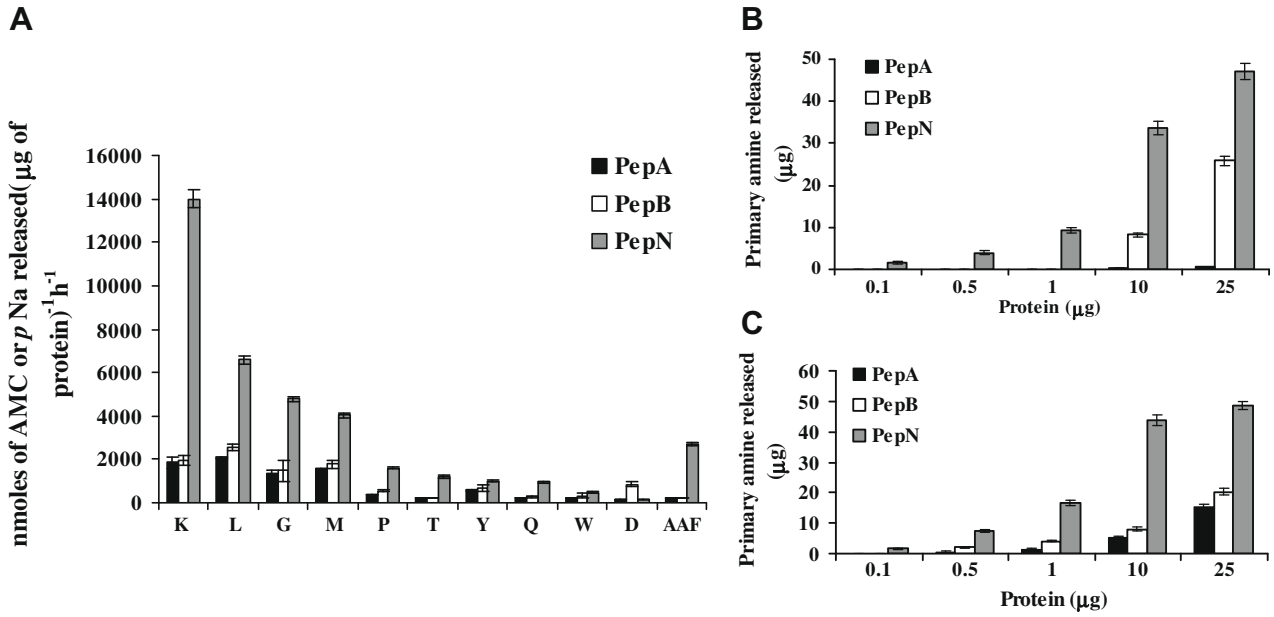

Fig. 3. Substrate specificity of PepA, PepB and PepN. Purified enzymes were assayed for their ability to hydrolyze a panel of 0.5 mM aminopeptidase substrates (A). Different amounts of purified enzymes were incubated at 37 °C with 100 µg of oxidized insulin B chain (B) or casein (C) for 4 h and newly released free amino groups were estimated. Data is representative of three independent enzyme preparations and shown as means ± SE.

strate AAF more efficiently than PepA or PepB. Also, PepN cleaved endopeptidase substrates [8]; however, PepA and PepB did not cleave these substrates (Supplementary Fig. 3). Further, kinetic studies were performed and Lys was the most favored substrate for PepN with the following profile: Lys > Leu > Gly/Met > AAF whereas PepA and PepB showed: Lys/Leu/Met > Gly > AAF. Importantly, the k_{cat} values of PepN for Lys, Gly, Met, Leu and AAF cleavage were ~3–15-fold higher compared to PepA and PepB.

Next, the cleavage of a peptide, oxidized insulin B chain, and a loosely folded protein, casein was studied. All three enzymes hydrolyzed casein in a concentration and time dependent manner (Fig. 3B and Supplementary Fig. 4). Notably, PepN was more efficient compared to PepA and PepB as cleavage was observed using lower amounts of enzyme. Interestingly, PepA was not able to cleave Insulin B chain. Also, increasing the time for cleavage did not enhance the ability of PepA to cleave Insulin B chain (Supplementary Fig. 4). Overall, these studies, using synthetic and natural substrates, showed that purified PepN possessed a greater catalytic ability compared to PepA and PepB. Also, this analysis showed that PepB possessed a broader substrate specificity compared to PepA.

3.4. Inhibitor and temperature stability analysis

These enzymes were further characterized using inhibitors and temperature stability studies. Purified PepA, PepB and PepN were incubated with different inhibitors and assayed for catalytic activity (Fig. 4A). All three enzymes were greatly inhibited by 1,10-phenanthroline and bestatin. Interestingly, PepA and PepB were more sensitive to pepstatin as compared to PepN. Next, the structural stability of these enzymes was analyzed using circular dichroism (CD). No major differences between the CD spectra of these three enzymes were observed (Supplementary Fig. 5); however, CD–T_m analysis clearly revealed that PepA was more stable at higher temperature (Fig. 4B). In fact, the melting temperature of PepA was 61 ± 0.8 °C whereas that of PepB and PepN was 55 ± 0.7 °C, using three independent enzyme preparations.

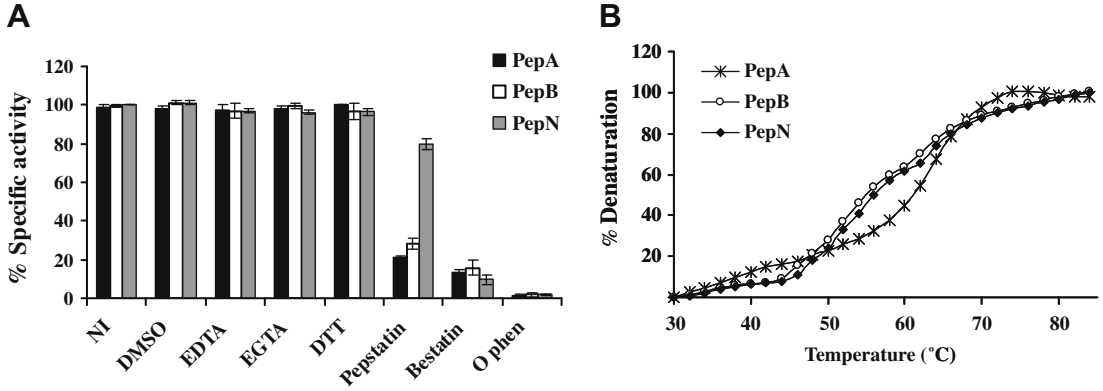

Fig. 4. Inhibitor and temperature stability studies on PepA, PepB and PepN. Purified enzymes were preincubated with different inhibitors for 15 min and cleavage of L-Leu-AMC was studied at 37 °C for 1 h. NI refers to the normalized activity of enzymes without any inhibitor. The data is shown as means ± SE of four independent enzyme preparations (A). The T_m of purified enzymes was measured by monitoring the CD at 222 nm with increase in temperature from 30 to 80 °C (B).

Table 1
Kinetic parameters of hydrolysis of selected substrates by PepA, PepB and PepN.

Substrates	PepA			PepB			PepN		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
L-Lys-*p*Na	220 ± 7	17.7	0.082	170 ± 4.5	14.3	0.089	382 ± 6	290	1
L-Leu-AMC	180 ± 6	17	0.091	140 ± 13	19	0.112	270 ± 7	90	0.33
L-Met-AMC	240 ± 6	20	0.081	140 ± 2	14	0.104	340 ± 19	80	0.235
L-Gly-AMC	140 ± 6	11	0.068	160 ± 9.5	12	0.071	310 ± 10	92	0.292
AAF-AMC	260 ± 20	10	0.034	330 ± 3	14.4	0.041	445 ± 12	53	0.126
L-Asp-AMC	ND	ND	ND	401 ± 2	11.7	0.023	ND	ND	ND

Different concentrations of substrates were subjected for hydrolysis by purified PepA, PepB and PepN. The kinetic parameters were calculated by direct linear plot. Data are representative of three independent enzyme preparations shown as means ± SE. ND indicates not detected.

4. Discussion

The highlight of this study is the comparative biochemical characterization of the two M17 family paralogs in *E. coli*. Although PepA and PepB are known as LAPs, this study clearly showed that they possess broad substrate specificity (Fig. 3) and cleave Leu as efficiently as Lys and Met (Table 1). Initially, the substrate cleavage pattern was analyzed by overexpressing PepA and PepB in Δ*pepN* (Fig. 1B). The increased catalytic activities upon overexpression of PepA or PepB were, most likely, responsible for better growth of Δ*pepN* during nutrient limitation and high temperature stress (Fig. 2). However, these effects observed upon overexpression of PepA or PepB were less compared to that observed upon PepN overexpression (Fig. 2). Most likely, this is due to the enhanced catalytic activity of PepN compared to PepA and PepB (Fig. 3 and Table 1). In fact, the extent of peptidase activities appears to directly correlate with growth of Δ*pepN* during nutritional downshift and high temperature stress.

In general, M1 and M17 members are sensitive to chelation with 1,10-phenanthroline, which chelates divalent cations like Fe^{2+}, Co^{2+} and Zn^{2+}, whereas differential sensitivity is observed with EDTA: a M17 member encoded by *Schizosaccharomyces pombe* is inhibited by EDTA [29] whereas lysine aminopeptidase, a M1 family member in *Aspergillus niger*, is less sensitive to EDTA and EGTA [30]. Studies with inhibitors demonstrated PepA, PepB and PepN to be sensitive to 1,10-phenanthroline and bestatin but not EDTA and EGTA (Fig. 4A). Interestingly, PepstatinA, an aspartyl protease inhibitor, inhibited PepA and PepB greater than PepN (Fig. 4A) probably due to the presence of Asp in the active sites of PepA and PepB but not PepN (Supplementary Figs. 6 and 7).

Distinct differences were observed in four properties of PepA and PepB. First, bioinformatics analysis showed that residues known to be involved in DNA-related activities of *E. coli* PepA are not conserved in PepB and most M17 orthologs (Supplementary Fig. 7). Second, PepA is larger than PepB, both in terms of subunit size and native molecular weight (Supplementary Fig. 1). Third, PepB, but not PepA, cleaved Asp (Fig. 3A). A previous study had shown that partially pure *E. coli* PepB preferred Glu over 5-fold more than Leu [31]. However, kinetic analysis showed purified PepB to cleave Asp less efficiently compared to Leu/Met/Lys/Gly (Table 1). The ability of PepB to cleave Asp at the N-terminal of peptide chain is due to the presence of a Lys residue present in the domain containing the active site [32]. As shown in Supplementary Fig. 7, this Lys residue is present only in *E. coli* PepB and *Vibrio cholerae* PepA but not other M17 family members. In addition, the CSATYRK motif is present only in PepB and Arg present in this motif may be interacting with N-terminus of acidic substrates [32]. Also, PepA was unable to cleave the oxidized insulin B chain peptide (Fig. 3B). Taking into account the ability to cleave Asp and insulin B chain, it appears that PepB has broader specificity compared to PepA. Fourth, CD–T_m analysis showed that the T_m for PepA was higher than PepB (Fig. 4B). This is in contrast to a previous study which showed that partially pure *S. typhimurium* PepB is stable at 70 °C [32]. In fact, our study agrees with another that showed PepA to be more resistant to heat denaturation compared to other L-Leu-*p*NA cleaving enzymes [33].

The broad specificity of PepN has been studied using structural biology approaches [34]. The substrate binding pocket in PepN is close to the active site and the top of it is composed of polar residues whereas the walls are made up of hydrophobic amino acids. This arrangement allows the binding of different amino acids which contributes to the broad specificity of PepN. Although, the crystal structure of PepB is not known that of PepA is known and the active sites are located in a cavity in the center of the hexamer that are accessed via three solvent channels [20]. PepA has two overlapping Zn^{2+} binding motifs and the metal binding motifs are rich in residues with carboxylate groups. The catalytically important residues (Supplementary Fig. 7) are conserved across all the orthologs and the catalytic Lys-282 in *E. coli* PepA (Lys-294 in mouse LAP) acts as a proton donor [20]. The crystal structure of *P. falciparum* LAP (PfA-M17) shows the substrate binding pocket interacting with P1 amino acids is narrow and majority of amino acids present at the entrance are hydrophobic which may be responsible for the restricted substrate profile with respect to polar and charged amino acids. In addition, the structure suggests difficulties for small amino acids to bind optimally at the substrate binding pocket [35]. Further studies are required to understand whether these differences are responsible for the lowered catalytic activity and differences in substrate specificity of PepA and PepB compared to PepN.

The current method of identifying the substrate specificities of peptidases relies on purification from natural or overexpressing systems. Consequently, this time consuming process reduces the number of enzymes that can be screened. On the other hand, using this strategy different peptidases can be cloned, expressed in Δ*pepN* and screened for activities in total cell lysates (Fig. 1A). After screening, only selected peptidases can be purified to confirm their substrate specificities. This aspect is important in light of the fact that ~2–5% of the genes encode for peptidases, irrespectively of the organism source [36]. Overall, implementation of this strategy can rapidly enhance the identification of peptidases with novel substrate specificities that may play important cellular roles and/or have industrial applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.142.

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