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# Biochemical and structural analysis of a site directed mutant of manganese dependent aminopeptidase P from *Streptomyces lavendulae*

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## ABSTRACT

Aminopeptidase P (APP) removes N-terminal amino acids from peptides and proteins when the penultimate residue is proline. To understand the structure-function relationships of aminopeptidase P of *Streptomyces lavendulae*, a conserved arginine residue was replaced with lysine (R453K) by site-directed mutagenesis. The overexpressed wild and mutant enzymes were of nearly 60 kDa and purified by nickel affinity chromatography. Kinetic analysis of R453K variant using Gly-Pro-*p*NA as the substrate revealed an increase in Km with a decrease in Vmax, leading to overall decrease in the catalytic efficiency, indicating that the guanidinium group of arginine plays an important role in substrate binding in APP. We constructed three dimensional models for the catalytic domains of wild and mutant enzyme and it revealed an interaction in R453 of native enzyme through hydrogen bonding with the adjacent residues making a substrate binding cavity whereas K453 did not participate in any hydrogen bonding. Hence, R453 in APP of *S. lavenduale* must be playing a critical role in the hydrolysis of the substrate.

**Key words:** Aminopeptidase P, catalytic domain, metalloprotease, site directed mutagenesis, *Streptomyces lavendulae*

## Introduction

Aminopeptidase P (APP, 3.4.11.9) cleaves the N-terminal amino acid residue from polypeptide chains where the second residue is proline. This activity is of interest since many biologically active peptides, including coagulants, enzymes, growth factors, hormones, kinins, neurotransmitters, and toxins, have an N-terminal Xaa-Pro sequence (Yaron, 1987). APP is known to be directly involved in the regulation of one such biologically active peptide, the vasodilatory hormone bradykinin, and inhibitors of human APP are promising cardiovascular therapeutic agents. Recently we have cloned the APP gene of *Streptomyces lavendulae* (NCBI Gen Bank Protein id: AGG68151.1) and found significant sequence similarity with other reported sequences (Nandan & Nampoothiri, 2014) and thus we predicted the overall structure of our protein is similar to that of other APPs.

*Escherichia coli* aminopeptidase P is a member of proline specific peptidases, catalyzes the hydrolysis of Xaa-Pro bond at the N-terminus of polypeptides (Yoshimoto et al., 1988; Yoshimoto et al., 1994). On the basis of information derived from the crystal structure, peptidase sequence alignments and the hydrolysis of organophosphate triesters of *E. coli* APP, active site residues such as Arg153, Arg370, Trp88, Tyr387 and Arg404 were identified as potential catalytic residues (Graham et al., 2003). Among these residues, we focused on Arg404 (Arg453 in *S. lavendulae* APP), which is participating in enzyme catalysis of *E. coli* APP (Jao et al., 2006).

Based on the previous study of the crystal structure of APP of *E. coli* (Graham et al., 2003) the arginine residue corresponding to Arg453 of *S. lavendulae* is thought to be involved in hydrogen bond formation with the carboxylate side chain of Asp260 and with the ring hydroxyl group of Tyr387 (Asp305 and Tyr435 in *S. lavendulae*). Thus, we predicted that replacement of this residue would result in a protein with altered catalytic rate, thus substantiating the role of this residue in *Streptomyces* APPs as well as reinforcing

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the structural similarity of *S. lavendulae* APP with other well studied APPs.

We constructed Arg453Lys (R453K), the site directed mutant having a lysine side chain to preserve the positive electrostatic environment. Based on the comparative kinetic study of the wild type and mutant enzyme, the mechanism underlying the alteration in activity is explained. Furthermore, the structural prediction using homology modeling was used to explore the general catalytic mechanism of APP.

## Materials and Methods

### *Chemicals*

All buffers and chemicals were purchased from Sigma, Aldrich. The restriction endonucleases and DNA polymerase were purchased from New England Biolabs. The QuikChange Multi Site-Directed Mutagenesis Kit was from Stratagene (USA). pET plasmids and *E. coli* strains were from Novagen (USA). Ni-NTA colums were purchased from Qiagen (Germany). Primers were synthesized by IDT (USA).

### *Sequence analysis*

In order to compare the protein sequence of *S. lavendulae* APP with other *Streptomyces*, *E. coli* and human APPs multiple sequence alignment was performed using Clustal W program (Thompson et al., 1997). The alignment was done to observe the sequence similarities and variations of catalytically important residues within these species. Further, amino acid alignment was also done with other *Streptomyces* species to compare the identity with *S. lavendulae* APP.

### *Site-directed mutagenesis of APP*

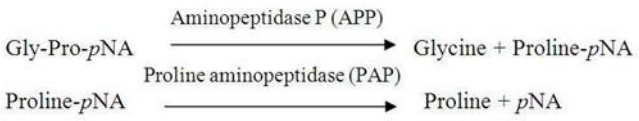
Mutation in the APP gene was generated using the QuikChange site-directed mutagenesis kit (Stratagene) using the primers (5'-gcggcatcggcgtcaagatcgaggacgaca-3' and antisense 5'-tgtcgtcctcgatcttgacgccgatgccgc-3'). PCR was performed under the following conditions: initial denaturation for 30s at 95°C, 16 cycles of 30s at 95°C, 1 min at 55°C and 10 min at 68°C. After digestion of the parental DNA for 1 h at 37°C with *DpnI*, the amplified plasmids were transformed into *E. coli* DH5α cells. The presence of mutation was confirmed by DNA sequencing.

### *Protein expression and purification*

Wild-type and mutant APP were over expressed as N-terminal His-tag fusion protein in BL21 (DE3) and purified as reported previously (Nandan & Nampoothiri, 2014). The purity of APP samples was confirmed by SDS-PAGE. Wild-type and mutant APP were used for comparative kinetic studies with peptide substrates.

### *Enzyme assay*

The enzyme reaction mixture composed of 1 mmol $l^{-1}$ substrate Gly-Pro-*p*NA and 50 µg of APP enzyme in 50 mmol $l^{-1}$ Tris-HCl buffer, pH 8.5. After 30 minutes of incubation at 37ºC, the enzyme reaction was stopped by heating at 100°C for 5 minutes, after cooling added 50 µl of proline aminopeptidase from *S. lavendulae* (0.5 units) and incubated at 37°C for 15 minutes and the amount of released paranitroanilde (chromogenic substrate) was measured at 405 nm. The steps involved in this coupled assay as follows as described by Yoshimoto et al. (1988).




### *Circular dichroism (CD) of APP and R453K APP mutant*

CD spectra were obtained from 200 to 260 nm at 0.5 nm intervals using a spectropolarimeter (Jasco J720, Japan) and a 0.2 cm cylindrical quartz cuvette. Protein samples were prepared at 0.1 mg/mL in 50 mM Tris HCl (pH 8.5). The spectrum for each sample was averaged over 10 acquisitions, and the background contributed by buffer was subtracted. The measured ellipticity data were converted into mean residue ellipticity, [θ], which is defined as [θ] = θ/10nCl, where θ is the measured ellipticity in milli degrees, l is the path length of the cuvette in centimeters, C is the molar concentration of enzyme subunits, and the number of residues per enzyme subunit. The CD data were plotted as mean residue ellipticity [θ] (degrees square centimeters per decimole) against wavelength in 1 nm steps.

### *Enzyme kinetics*

The kinetic parameters such as $K_m$, $V_{max}$ of the purified mutant enzyme were estimated for Gly-Pro *p*NA using concentrations ranging from 0.2 to 5 mmol $l^{-1}$. 50 µg APP mutant proteins was used for this study. Activity was continuously measured at 37°C as described above. Kinetics parameters were measured with Graph Pad Prism version 6 for Windows (Graph Pad Software, San Diego, CA, U.S.A; www.graphpad.com). For $K_m$ determination, the data were plotted to fit the Michaelis–Menten equation by non-linear regression.

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***Effect of metal ions on R453K mutant***

Metal ions $Mn^{2+}$ and $Co^{2+}$ were selected in the range of 0.1 to 1.5 mM on the basis of the results of previous study (Nandan & Nampoothiri, 2014). The apoenzyme of APP and R453K mutant was prepared and incubated with these metal ions separately and enzyme assay was performed to compare the effect of these metals on mutant enzyme.

***Structural model of APP***

The sequence of the primary structure of S. *lavendulae* APP (NCBI Gen Bank Protein Id: AGG68151.1) was aligned with the sequence of putative aminopeptidase P from *Bacillus anthracis* (Protein Data Bank id: 3ig4). The alignment data were submitted to SWISS-MODEL (Arnold et al., 2006; Schwede et al., 2003; Guex & Peitsch, 1997) for generation of a homology model of APP using Swiss-Pdb Viewer 3.7 as the interface. The structural model obtained was analyzed using Swiss-Pdb Viewer. The resulting 3D model was then subjected to another refinement procedure including several rounds of energy minimization (until convergence) and short molecular dynamics runs. This process was carried out to check the stability of the model over time. By homology modeling, three-dimensional models were constructed for the catalytic domains of APP.

## Results

***Multiple sequence alignment of APP- Catalytic residues are highly conserved***

*S. lavendulae* APP sequence was aligned with the sequences of *S. coelicolor*, *E. coli* and human APP (Figure 1A). The X ray crystal structure of *E. coli* has been revealed the important residues of catalysis such as H243, H350, H361, R404, Tyr387, Asp260 and Trp88 (Wilce et al., 1998) corresponding to H288, H396, H407, R453, Tyr435, Asp305 and Trp140 residues of *S. lavendulae*.

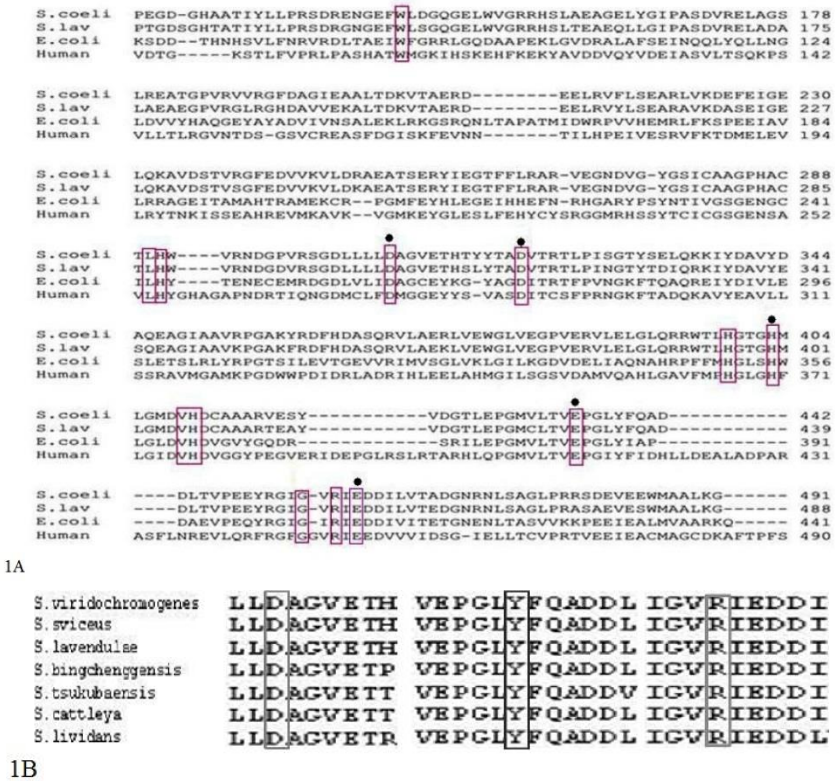


**Figure 1. (A)** *Alignment of entire ORF sequences of APPs from S. coelicolor, S. lavendulae, E. coli and human. Multiple sequence alignments were performed using the CLUSTAL algorithm. The amino acid residues involved in catalysis are marked by boxes and those involved in metal coordination are indicated by black dots.* **(B)** *Aligned APP sequences of various Streptomyces stains. Conserved residues of interest Asp (D), Tyr (Y) and Arg (R) described are indicated in boxes.*

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Recent structural studies and electron paramagnetic resonance investigations of APP have shown that the $MnA^{2+}$--$MnB^{2+}$ binuclear manganese cluster, located in the C-terminal domain, is critical for maximal catalytic activity of the enzyme (Yaron & Mlynar, 1968; Yoshimoto et al., 1988; Bazan et al., 1994; Cunningham & O'Connor , 1997; Wilce et al., 1998; Zhang et al., 1998; Lowther & Matthews, 2002) The residues involved in metal ion ligands in *E. coli* are Asp271, Glu383, His354, Glu406, Asp260, Asp271 corresponding to Asp317, Glu431, His400, Glu455, Asp305, Asp317 in *S. lavendulae* (Figure 1A). The role of Arg 404 in *E. coli* APP is explained by Jao et al., (2006). Among the *Streptomyces* species the conserved residues in APP were identified and marked in Figure 1B.

***Overexpression and purification of site-directed mutant of APP (R453K)***

During overexpression in *E. coli* BL21 (DE3), the mutant enzyme was expressed as soluble fraction as in the case of wild type. Mutant protein was purified through Ni-NTA column and was detected as ≈ 60 kDa on 12% SDS-PAGE similar to the wild type enzyme (Figure 2).

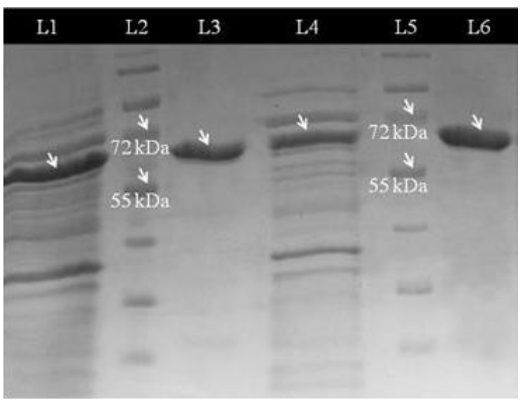


**Figure 2.** *SDS-PAGE showing protein expression. L2 and L5- Protein markers; L1- APP overexpression; L3- Purified APP; L4- R453K overexpression; L6- Purified R453K.*

***Kinetics of mutant R453K***

We also characterize the kinetics of mutant and compared the values with that of wild type. The $V_{max}$ and $K_m$ and values are 0.3953 µmol $min^{-1}$ and 1.593 mmol $l^{-1}$ respectively. The $K_m$ value of mutant was higher than that of wild type (0.4697 mmol $l^{-1}$) and it indicates the less affinity of mutant protein towards the substrate Gly-Pro *p*NA.

***Metal replacement studies***

Effect of increasing concentrations of $Mn^{2+}$ and $Co^{2+}$ on the hydrolysis of Gly-Pro by wild type APP and mutant APP was examined (Figure 3). $Mn^{2+}$ was seen to promote activity of both the enzymes, with maximal activity observed at 0.8 mM. Assays of APP wild type protein containing $Co^{2+}$ enhanced the enzyme activity at 1 mM concentration while no such activation was observed in the case of mutant enzyme. Only $Mn^{2+}$ could enhance the activity of mutant protein but not $Co^{2+}$.

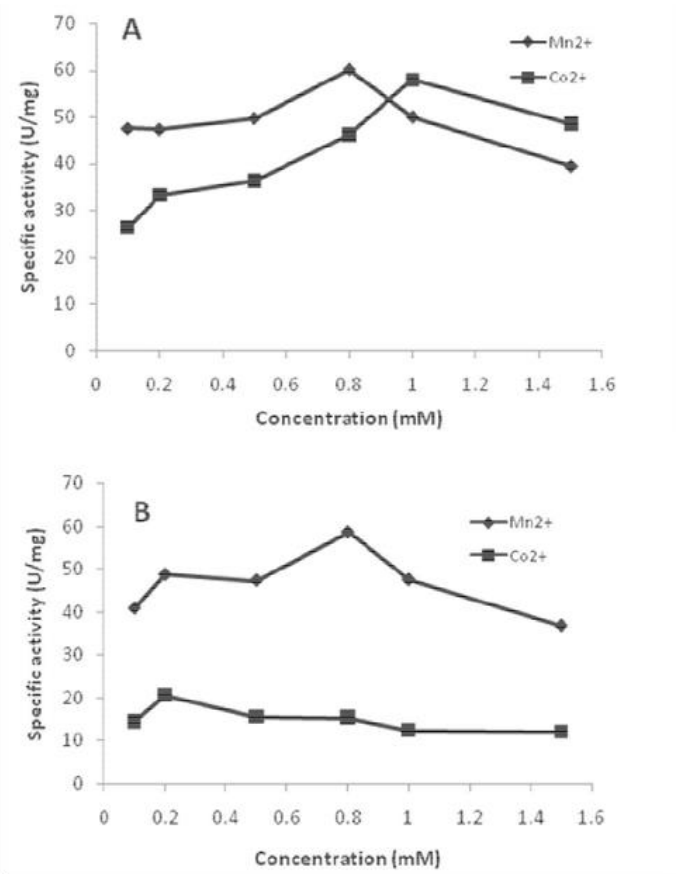


**Figure 3.** *Effect of $Mn^{2+}$ and $Co^{2+}$ on enzyme activity* ***(A)*** *APP* ***(B)*** *in R453K mutant.*

***Circular Dichroism (CD) spectra of purified APP and R453K mutant***

CD spectra were obtained for wild type APP and mutant R453K to determine whether the secondary structure of the native enzyme was retained in the mutant. No major differences were detected in CD spectra measured from 200 to 260 nm, indicating that no substantial changes in the

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backbone fold had resulted from the single-amino acid substitutions made in the respective mutant (Figure 4).

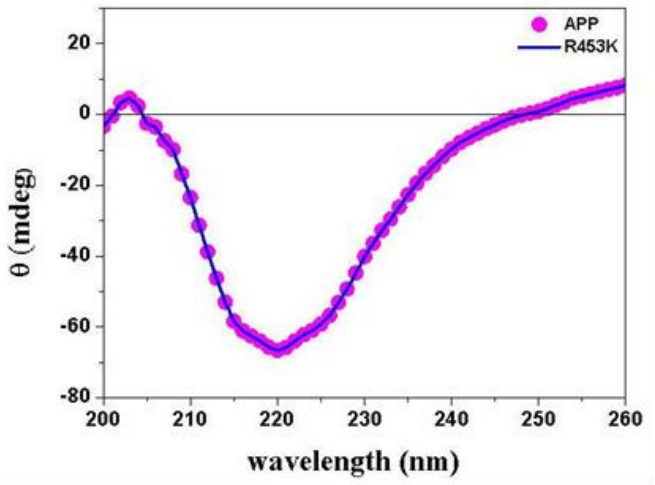


**Figure 4.** *CD spectra of APP and R453K mutant.*

***Three dimensional modeling of APP from S. lavendula***

Knowledge of the three-dimensional structure of a protein is essential to gain insight into its cellular function. The difficulties encountered in obtaining crystals with a three-dimensional net that was ordered and stable enough to be studied led to the use of various algorithms and computer packages to predict the structure of a protein from its amino acid sequence, which is possible, provided the three-dimensional structure of a homologous protein with sequence similarity has been previously resolved.

To characterize the structural requirements for APP activity and the specificity of *S. lavendulae*, three dimensional models were constructed based on the APP crystal structure of *Bacillus anthracis* (PDB id: 3ig4) (Figure 5) which was generated using SWISS MODEL. The N-terminal domain (residues 1 to 166) is joined by a short helical linker (residues 167 to 174) to the larger C-terminal domain (residues 175 to 488). The dinuclear manganese (II) centre is located in a depression on the inner concave surface of the sheet of the C-terminal domain.

Similarly, a three dimensional model of the active site of *Streptomyces* APP was constructed (Figure 6). The organization of the active site obtained after refining the model was very similar to that observed in other APPs.

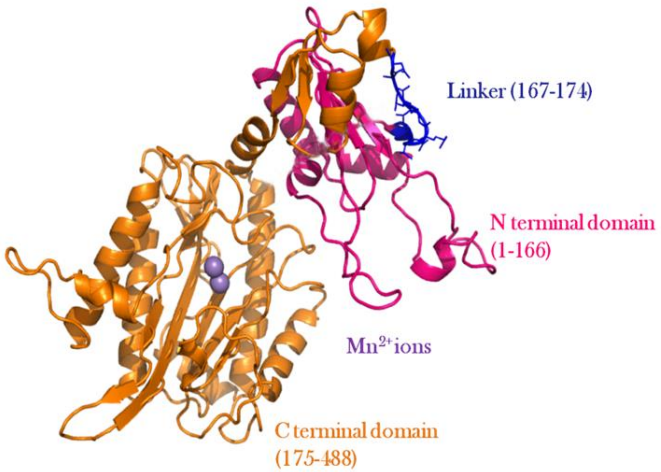


**Figure 5.** *Structure of S. lavendulae APP. Stereoview of one monomer representing the C-terminal, N-terminal and linker region. The $Mn^{2+}$ ions are represented by violet spheres in the catalytic center.*

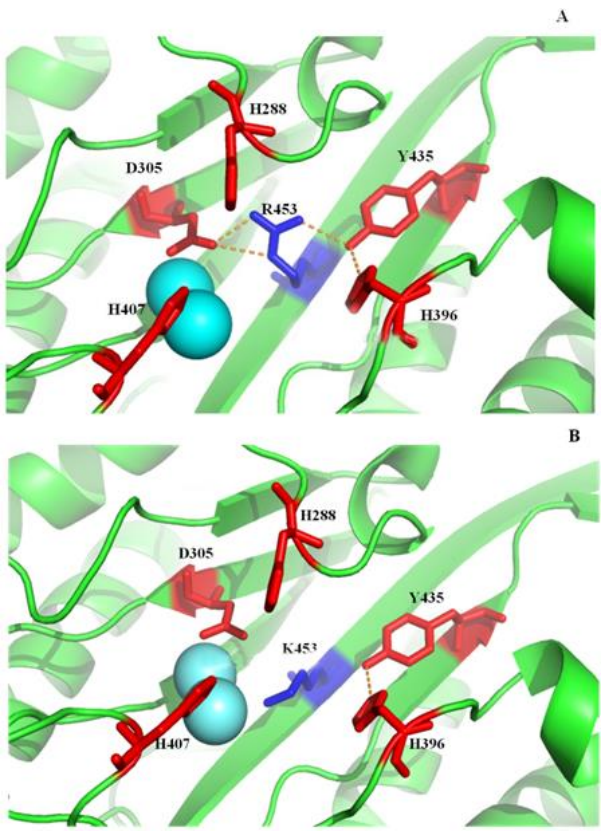


**Figure 6.** *Comparison of active site organization of APP and R453K mutant protein. The active site of S. lavendulae APP* ***(A)*** *wild type* ***(B)*** *mutant R453K. The mutated residue is shown in blue.*

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In a study by Jao et al., (2006) reported that the positively charged guanidinium side chain of Arg404 in *E. coli* APP (*S. lavendulae* Arg453) is properly positioned to engage in hydrogen bond formation with carboxylate side chain of Asp260 (*S. lavendulae* Asp305) and with the ring hydroxyl group of Tyr387 (*S. lavendulae* Tyr435). Interestingly, this hydrogen bond network is absent in mutant protein (Figure 7).

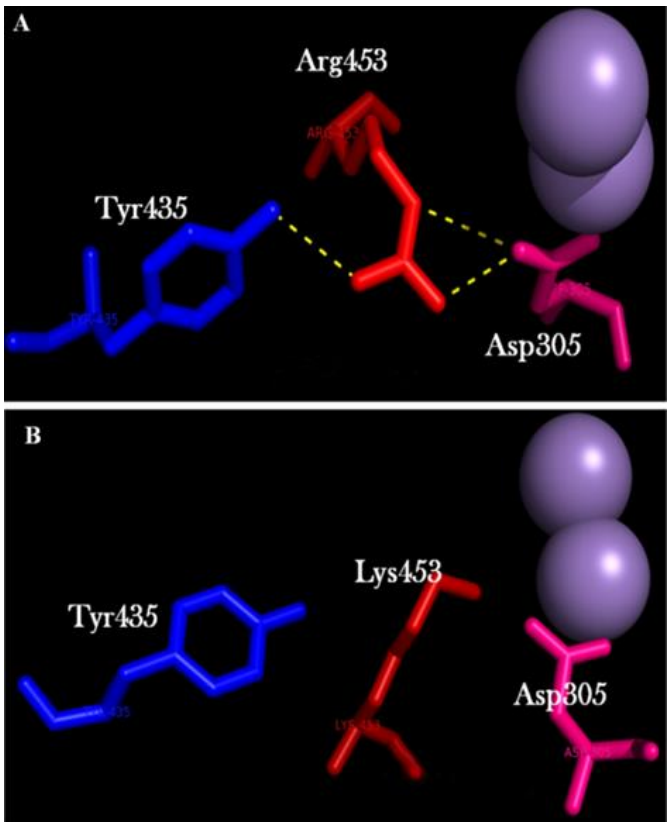


**Figure 7.** *Comparison of the orientation of the hydrogen bond network in the active site of APP formed by Asp305, Arg453 and Tyr435.* ***(A)*** *The guanidinium group of Arg453 interacting with the carboxylate of Asp305 and the hydroxyl group of Tyr435 in the wild-type APP* ***(B)*** *the modeled structure displaying the relative position of R453K, showing the absence of hydrogen bond interactions with two neighboring residues. The hydrogen bonding interactions are shown as dashed yellow lines.*

The metal ions in APP are interacting with particular residues in the catalytic sites through metal-ligand interactions. From the alignment, the metal coordinating ligands of *S. lavendulae* APP are predicted to be Asp305, Asp317, His400, Glu431 and Glu455. All the residues interacting with the ligand are completely conserved between model and template. These interactions are represented by LigPlot software (http://www.ebi.ac.uk/thornton-srv/software/LIGPLOT) (Figure 8). The analysis indicated that the residues in the metal ligand formation are not get altered by the mutation created. Thus, the residues predicted for the ligand formation in wild type as well as the mutant are the same.

## Discussion

We constructed APP mutant, R453K, having a lysine side chain to preserve the positive electrostatic environment, to study the role of Arg453 of *S. lavendulae* APP catalysis.

The structure of *E. coli* APP has been solved previously in complex with the product dipeptide Pro-Leu and the substrate-like inhibitor apstatin (Wilce at al 1998; Graham et al., 2004). These studies identified the substrate binding pocket of APP of *E. coli*: the S1 site (Tyr229, His243, Asp260, Asp271, His354, Val360, and His361); the S1′ site (Leu242, His243, Asp260, His350, Glu383, and Arg404); and the S2′ site (Arg153, Arg370, Tyr366, Gly351, and His354). The sites are defined according to the nomenclature of Schechter and Berger (Schechter & Berger, 1967). S1′ is the site occupied by the Pro residue of the substrate. We have targeted our mutation site on Arg404 (Arg453 in *S. lavendulae*) as it is occupied in the S1′ site of substrate binding.

Early studies on naturally abundant APP purified from *E. coli* showed that the enzyme is activated optimally by $Mn^{2+}$, and weakly by $Co^{2+}$, $Ni^{2+}$, and $Cd^{2+}$ (Yaron & Berger, 1970). Recombinant *E. coli* APP has been reported to be fully activated by $Mn^{2+}$ and weakly by trace amounts of $Zn^{2+}$ (Yoshimoto et al., 1989). Human cytosolic APP and membrane-bound rat lung APP are also activated strongly by $Mn^{2+}$, less activated by $Co^{2+}$, and not at all by $Ca^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ (Rusu & Yaron, 1992; Hooper et al., 1992; ; Orawski & Simmons, 1995). Spectroscopic measurements on *E. coli* APP are consistent with a substantial difference between the affinities of the enzyme for the first and second $Mn^{2+}$atoms of the dinuclear cluster (Zhang et al., 1998), and recombinant human cytosolic APP expressed in *E. coli* is active when it contains only one molar equivalent of $Mn^{2+}$ (Cottrell et al., 2000). The situation is complicated further by an observation that porcine membrane-bound APP is activated by one molar equivalent of $Zn^{2+}$ (Hooper et al., 1992). It is unlikely that the reported differences among the

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cited APP arise from different active site clusters, since an alignment of APP sequences shows that the metal-binding residues are strictly conserved (Graham et al., 2004). In *E. coli* APP, the residues which are involved in the metal ligand interaction are identified as Asp260, Asp271, His354, Glu383 and Glu406 (Graham et al., 2005).

Inductively coupled plasma mass spectroscopy and circular dichroism spectroscopy showed that Arg404 is not required for stabilization of protein secondary structure (Jao et al., 2006). In our study we observed that only $Mn^{2+}$ could enhance the activity of both wild and mutant enzyme, but the addition of $Co^{2+}$ can enhance only the wild type protein but not the mutant protein. An earlier report by Graham et al., showed significant activity enhancement of *E. coli* APP in the presence of $Mn^{2+}$ (Graham et al., 2005).

As predicted from sequence alignment (Roderick & Matthews, 1993) the C-terminal catalytic domain of APP is structurally related to two other peptidases: the Xaa-Pro dipeptide-specific enzyme prolidase (Bazan et al., 1994) and the Met-Xaa- oligopeptide-specific enzyme methionine aminopeptidase (Roderick & Matthews, 1993). Based on their common fold, these enzymes are referred to as the "pita bread" metalloenzymes. Since they all share the same set of conserved active site residues with two divalent metal ions bound to the same set of ligands and act upon similar substrates, it had been proposed that they share a conserved catalytic mechanism (Lowther & Matthews, 2002).

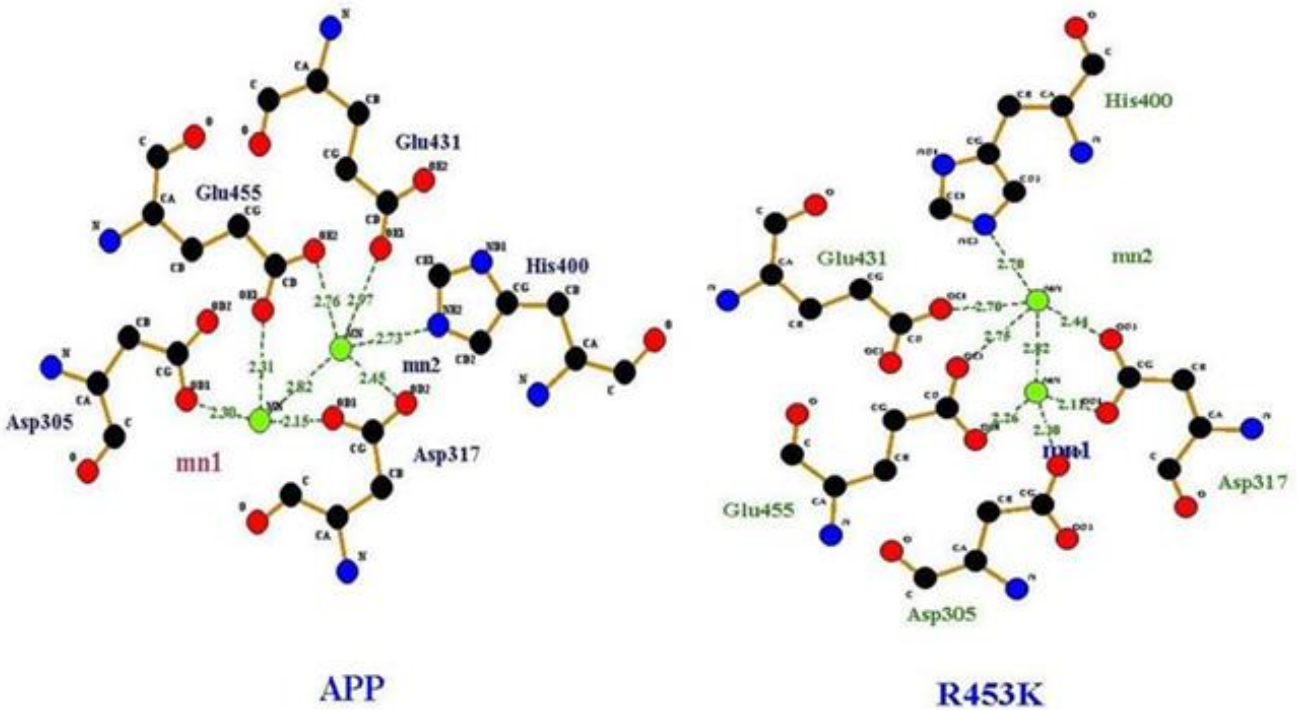


**Figure 8.** *LigPlot representation of APP and APP mutant (R453K). The Mn2+ ions are represented by green spheres. Hydrogen bond interactions are represented by green dashed lines.*

## Conclusion

In the present study, we demonstrated that a single mutation at catalytic position 453 can alter the activity of APP in hydrolyzing the substrates with Xaa-Pro bonds. The model of the active site of *S. lavendulae* APP conserves all the known features of other members of this peptidase clan and predicts Arg453 as a critical active site residue. Since the supplementation of Mn2+ regains the activity of mutant APP it can be concluded that Arg453 is not required for metal chelation but unlike the wild type enzyme, the mutant protein couldn't hold $Co^{2+}$ as a metal ion for catalysis. Circular dichroism spectral analysis and predict protein analysis showed there is no substantial change in the secondary structure of protein due to this mutation and thus Arg453 may

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not require for the stabilization of protein secondary structure. The hydrogen bond interaction between Arg 453 to Tyr 435 and Asp 305 is suggested by the reduced catalytic efficiency of the R453K mutant. The residues (Asp305, Asp317, His400, Glu431 and Glu455) which are likely involved in the metal ligand formation are predicted by LigPlot. By considering all the structural evidences available, it can be concluded that S. *lavendulae* APP is a homotetramer aminopeptidase belonging to the M24 peptidase family. However, to develop *Streptomyces* aminopeptidases as an excellent biocatalyst, further studies on other amino acid residues related to enzymatic performance are needed.

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