

Purification and Characterization of Cyclophilin-a Proteins That Associated With Protein Folding in Salmonella Typhimurium

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Abstract

Salmonella Typhimurium (ST) is the zoonotic pathogenic Gram-negative bacteria to causes infectious disease in humans as well as in animals. It causes gastrointestinal illness and fever called salmonellosis, which is foodborne diarrheal and leading cause of millions of deaths worldwide. *Salmonella* enterica serovar Typhimurium (S. Typhimurium) during its pathogenesis takeover the actin cytoskeleton of their host cells and this is the crucial step of its infection cycle. Cyclophilin A, a type of peptidyl-prolyl isomerase that's encoded by the *ppiA* gene in ST, plays pleiotropic roles in maintaining bacterial physiology. In this research, the proteomic characterization of the peptidyl-prolyl *cis-trans* isomerase- A (Cyclophilin A) from *Salmonella* Typhimurium is reported. Cyclophilin A (CypA) protein from *Salmonella* Typhimurium proved to be a highly conserved protein sequence and highly homologous compared to other organisms. This protein was expressed in *Escherichia coli* and then purified in a recombinant form protein exhibited a characteristic PPlases activity ($V_{max} = 0.8752 \pm 0.13892 \mu\text{moles/ min}$, $K_m = 0.9315 \pm 0.5670 \mu\text{M}$) in comparison to control. Also, in this study the mass spectrometry analysis of Cyp A protein-peptide showed the highest sequence similarity with the cyclophilin protein of *Salmonella*. PPlases proteins enzyme data suggest that Ppi-A has roles in the protein folding that may be contributing to the virulence of *Salmonella* by isomerization of protein outline. These results suggest an active and vital role of this protein in protein folding along with regulation in *Salmonella* Typhimurium.

Introduction

Salmonella enterica serovar Typhimurium (ST) is one of the most common pathogenic Gram-negative, non-typhoidal bacteria causing foodborne diarrheal and salmonellosis (NTS) disease in humans. *Salmonella* Typhimurium is one of the widespread causative agents of human salmonellosis in the world and is among the top five most detected pathogens for each major food from animal species (Rabsch et al. 2001). As per CDC zoonotic diseases database, human salmonellosis causing by *Salmonella* Typhimurium is one of the most important in the world (Sabirovic et al. 2010). The global burden of foodborne *Salmonella* estimated around 93.8 million cases reported worldwide, out of which the disease claims 0.15 million lives (Majowicz et al. 2010). The common virulence strategy of the *Salmonella* genus is adhesion, invasion, intracellular replication and bacterial dissemination from the intestinal cells of the host. The ability of bacteria to sense and respond to unfavorable changes in the host environment is important for their survival and infection (Foster and Spector 1995).

Peptidyl-prolyl isomerase (PPlases) a family of evolutionarily conserved enzymes, initially discovered in eukaryotes, catalyzes a diverse process required for protein folding at proline residues, which accelerates the rate of exchange between *cis* and *trans* isomers (Fischer et al. 1998). The catalysis of the *cis-trans* isomerization of peptidyl-prolyl bonds done by the PPlases enzyme (Lang et al. 1987). PPlase includes four different families unrelated in their amino acid sequences based on drug resistance and homology. These are cyclosporin A (CsA)-binding proteins, cyclophilins, the FK506 and rapamycin binding proteins, FKBP, parvulins which do not bind immunosuppressant drugs (Fischer et al. 1998; Schiene-Fischer et al. 2013). Members of the cyclophilin family are also called immunophilins

because they are involved in intermediate effects of immunosuppressive drugs, Cyclosporine A (Hamilton and Steiner 1998).

Peptidyl-prolyl isomerase protein is ubiquitously distributed in almost all living organisms, from bacteria, plants to humans. Bacterial PPIases have homologs with membrane-associated lipoproteins which involve post-translocation secretion, protein folding and stability (Sarvas et al. 2004; Unal and Steinert 2014). *Salmonella* Typhimurium has identified peptidyl-prolyl *cis-trans* isomerase called "rotamase"; a homolog of human cyclophilins. Protein Cyclophilin A (CypA) is widespread in the cytosol and secretory protein that belongs to a family of evolutionary protein preserved *cis-trans* isomerases (PPIases) that catalyze protein folding at prolyl amino acid and cellular trafficking (Nigro et al. 2013). Cyclophilin shows enzymatic activity required for optimal protein folding and it also plays a vital role in multiple bacterial systems like the stress response, infectivity and virulence factors. Cyclophilin binds with high affinity to the clinically important immunosuppressive agent cyclosporin A (CsA) (Moochhala and Renton 1986).

These PPIase plays an important role in protein conformation, which occurs during biological conditions like refolding of denatured proteins (Schiene-Fischer and Yu 2001). PPIase enzymes were initially thought to be limited to folding proteins only, but recent research showed their other biological functions like signal transduction (Walsh et al. 1992), intracellular trafficking (Wintermeyer et al. 1995), gene transcription (Hanes 2015), cell cycle regulation (Baum et al. 2009), refolding of aggregated proteins (Zhang et al. 2013), regulation of reactive oxygen species (ROS) (Linard et al. 2009), apoptosis (Ding and Nam Ong 2003), proliferation and transformation (Zhu et al. 2015) and function as a molecular timer (Lu et al. 2007).

In the bacterial membrane envelope, PPIases also play essential and various biological functions. In *Salmonella*, SurA protein (PPIases type protein) found in the outer membrane play role to maintain the maturation of outer membrane proteins and resistance to various stress agents (like ROS and RNS), because it is sustain the outer membrane protein biogenesis and assembly (Behrens-Kneip 2010). Other protein like SurA from *E. coli*, *Shigella flexneri*, and *Salmonella* Typhimurium also help to cell adhesion and invasion of (Sydenham et al. 2000). In *E. coli*, PpiB also controls cell division by modulating the function of various proteins which are directly or indirectly associated with the cell division (Skagia et al. 2017). In this study, the role of Peptidyl-prolyl *cis-trans* isomerase- A type of cyclosporin is explored. Expression and purification of recombinant STPpiA protein to check the enzymatic activity was used and confirmed by the mass spectrometry sequencing. Finally, we demonstrate that STPpiA is an active type protein that illustrates the function of PPIases, which could be a regulator to facilitate the survival and virulence factors of bacteria. Furthermore, the exact biological role and effects of the STPpiA function in *Salmonella* systems remain a challenge for future studies. Thus, this study shows the importance of STPpiA for virulence of *Salmonella* Typhimurium and how this protein may be targeted to sensitize *Salmonella* Typhimurium to virulence regimes.

Materials And Methods

Bacterial strains and plasmids

The *E. coli* strain DH5 α and T7 Express lys γ were used for cloning and protein expression purposes. *E. coli* strains were cultured in Luria broth (LB). Kanamycin (Sigma) was added, where necessary, to LB medium at a concentration of 30 $\mu\text{g}/\text{mL}$.

Expression and Purification of STPpiA Proteins

The recombinant clone PpiA_pET28c construct and conformation was done by double digestion and PCR amplification of STPpiA from the recombinant clone PpiA_pET28c plasmid. The recombinant PpiA_pET28c construct was transformed into competent *E. coli* T7 Express lys cells. Finally, 50 μl of the re-suspended pellets were plated on kanamycin (30 $\mu\text{g}/\text{ml}$) plus chloramphenicol (10 $\mu\text{g}/\text{ml}$) containing LB agar plate and incubated at 37 $^{\circ}\text{C}$ overnight.

Then the isolated colonies were inoculated and grown overnight in LB broth containing antibiotics at 37 $^{\circ}\text{C}$ at 180 rpm. The cultures were diluted (1:100) in fresh media and grew at 37 $^{\circ}\text{C}$, 108 rpm ($\sim 3\frac{1}{2}$ hrs) to an OD₆₀₀ of 0.5. About one ml of this culture was removed in a 1.5 ml tube as an un-induced culture. The remaining culture was induced using isopropyl- β -D-thiogalactopyranoside (IPTG) final concentration 1.0 mM. Both un-induced and induced cultures were kept at 30 $^{\circ}\text{C}$, 180 rpm overnight the cells were harvested by centrifugation 7000 x g at 4 $^{\circ}\text{C}$, and the pellet was stored at -80 $^{\circ}\text{C}$.

The recombinant protein STPpiA was purified using the Ni-NTA affinity chromatography (Qiagen). The bacterial pellet was thawed on ice and suspended in a 10 ml chilled lysis buffer containing 50 mM sodium phosphate pH-8.0, 150 mM sodium chloride and 10mM final imidazole concentrations. After that lysozyme was added (1 mg/ml final concentration) to suspension and incubated on ice for 30 min. The cells were also lysed by 15 cycles of sonication in ice. Each cycle has a 45-second pulse of amplitude 48 Hz and a 45-second gap before the next pulse. PMSF as protease inhibitor 1 mg/ml final concentration was added to the cell suspension.

The lysate was centrifuged (10000 g, 4 $^{\circ}\text{C}$, 15 minutes) and the supernatant was filtered through a 0.45 μm syringe filter. The Ni-NTA column was equilibrated with 20 volumes of lysis buffer. The cell-free supernatant obtained by centrifugation was loaded in the Ni-NTA column. The unbound proteins from column were removed by washing 50 mM sodium phosphate (pH 8.0), 150 mM sodium chloride and 40 mM imidazole final concentrations. The protein bound to the Ni-NTA column was eluted with an elution buffer containing 50 mM sodium phosphate (pH 8.0), 150 mM sodium chloride and 200 mM imidazole final concentrations. The fractions were analyzed by spectrophotometer at 280 nm, and high absorbance peak fractions were pooled and dialyzed against 50 mM sodium phosphate (pH 7.5) buffer at 4 $^{\circ}\text{C}$. The purity of eluted fractions was then analyzed by SDS-PAGE and total protein was estimated by Bradford's total protein assay method using the BSA as standard. Finally obtained protein samples were stored at -80 $^{\circ}\text{C}$ for further experiments.

Western blotting investigation of recombinant proteins:

After the purification, STPpiA protein was resolved on SDS-PAGE followed by transferring onto polyvinylidene difluoride (PVDF) (0.2 μ M) membrane (200 mA for 2 hrs). The PVDF membrane was blocked with 5 % skimmed milk in PBST overnight at 4 °C. After that the membrane was washed with PBS and 0.05 % Tween 20 (PBST) solution. After washing with PBST, the membrane was incubated with a 1:1000 dilution mouse anti-His antibody at 37 °C for 3 hrs. After washing, the blot was incubated with 1:15000 alkaline phosphatase-conjugated anti-rabbit IgG at 37 °C for 2 hrs; which acted as the secondary antibody. Then blott membrane was developed using enzyme substrates like 5-Bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium chloride.

Identification of proteins by Mass spectrometry

The purified STPpiA protein was resolved on SDS- PAGE and was stained with CBB stain. After destining with 10 % methanol and 10 % acetic acid solution, the protein band was cut carefully and digested by trypsin followed by LC-MS, MS/MS was done to record mass spectrum (MS). The mass spectrometric data were analyzed manually and with the help of the MASCOT search engine (www.matrixscience.com). The highest score peptides sequences were submitted to NCBI protein BLAST.

PPlases enzyme activity by recombinant proteins:

There have been several assays devised to measure PPlases activity based on Fisher 1990(Fischer and Schmid 1990). The assay is based on the little difference in absorbance determined for the *cis* and *trans* isomers of Suc-Ala-Xaa-Pro-Phe-4-nitroanilide. To estimate enzyme activity we use substrate concentration from 0.25 to 10 μ M N-succinyl-ala-ala-pro-phe-p-nitroanilidine as test peptide, assay buffer [50 mM HEPES (pH 7.8) and 150 mM NaCl prechilled] and 300 μ g of the recombinant PPlase protein makeup 1 ml final volume. The reaction was initiated by the addition of chymotrypsin (500 μ g/ml) and the change in absorbance at 390 nm was monitored using a spectrophotometer (Perkin-Elmer Lambda Bio20) for 3 min at 25 °C. The enzyme kinetic analysis and the relation between velocity (v) substrate concentrations (S) were done through the Michaelis-Menten equation.

Results

Purification of recombinant proteins STPpiA

The expression of recombinant protein STPpiA was done using the transformed PpiA_pET28c plasmid into the T7 Express *lys γ* strain of *E. coli*. The induction of the recombinant STPpiA protein was confirmed by SDS-PAGE followed by CBB R-250 staining as shown in Fig. 1A. The full-length recombinant STPpiA protein was purified from the PpiA_pET28 containing *E. coli* culture using affinity chromatography by using nickel-NTA resin. A highly purified preparation of STPpiA was recovered from the column by elution with 500mM imidazole. The recombinant STPpiA protein had an apparent size of ~ 24kDa on SDS-PAGE gel (Fig.1A) and reacted with anti-His antibody and showed ~24kDa on western blot (Fig.1B). A single band with good intensity was observed on the membrane suggesting the recombinant STPpiA protein

with Histidine-tag. The size of the detected band was ~24kDa with a Histidine Tag (~ 3 kDa), which predicted the molecular weight for STPpiA protein as ~21kDa.

Identification of proteins by Mass spectrometry

After the molecular weight confirmation by SDS-PAGE and Western blot analysis, the purified recombinant STPpiA was verified by using the proteomic analysis with liquid chromatography-mass spectrometry. The full mass spectrum, as shown in Fig. 2a and peaks were marked with corresponding peaks of STPpiA peptides (Fig. 2a and b). The LC-MS and MS/MS were subjected to the MASCOT database search and identified the target protein band with a higher significance score. The result showed the identification of some Cyclophilin A-type PPlases isoforms. The peptide sequence coverage observed was more than 41 %. Detailed peptide coverage information was provided in bold (Fig. 3). The LC-MS analyses strongly suggest the presence of recombinant STPpiA protein. These peptides matched the original sequence from NCBI, indicating the correct protein identification (Fig. 3a). The study of LC-MS data strongly suggests the presence of the recombinant protein STPpiA. Such peptides matched the original NCBI sequence, suggesting the appropriate identification of the proteins (Fig. 3b).

Enzymatic activity of the purified STPpiA protein

After the confirmed identification of the protein by LC-MS and MS/MS analysis, we determined the functional (enzymatic) activity of this purified and dialyzed recombinant protein. The velocity (v) of an enzyme-catalyzed reaction was observed to be hyperbolically related to the substrate concentration (S) through the well-known equation of Michaelis-Menten ($v = V_{max} [S] / (K_m + [S])$). The purified STPpiA protein exhibited PPlases enzymatic activity and showed $V_m = 0.8752 \pm 0.13892 \mu\text{moles/ min}$, $K_m = 0.9315 \pm 0.5670 \mu\text{Min}$ comparison to control (Fig. 4). The purified protein found to be a first-order rate constant, and the presence of purified STPpiA showed an increase with an increase in the protein concentration (Fig. 4), thus, implying that the STPpiA specifically contributed to the observed PPlases activity.

Discussion

Salmonella Typhimurium is one of the major serovars commonly isolated from the human gastrointestinal tract and cause of gastrointestinal infections. The PPlases proteins are associated with a broad range of pathologies in various pathogenic bacteria. The initial function known only to cyclophilin was participation in only protein folding. Among the PPlases, CypA appears to play a pivotal role in the proper protein folding in many biological conditions. Studies showed that ked CypA expression to several cellular processes that require actin polymerization and cytoskeletal remodeling (Calhoun 2008; Saleh et al. 2016). Cyclophilin protein also plays a role as an antifungal, antiviral, and antioxidant agent (Wong et al. 2010). The recombinant from of cyclophilin from *Pyropia yezoensis* express and purified in *E. coli* showed cell proliferation (Jung et al. 2019). Some studies showed that PPlases are very essential for proteins function/folding in bacterial/viral infection and diseases (Theuerkorn et al. 2011; Unal and Steinert 2014; ZHANG et al. 2014). In the pathogenic *Brucella*

cyclophilins are also playing a vital role in virulence and survival in the host cells (Roset et al. 2013). In *Staphylococcus aureus* (Wiemels et al. 2017) and *Enterococcus faecalis* (Reffuveille et al. 2012) PPIases also play an important role in the secretion of virulence factors. In *E. coli* are combined protein Cyclophilin from *Trypanosoma cruzi* expressed and the purified recombinant protein exhibited PPIases activity (Bua et al. 2001). Some bacterial cyclophilins also play important chaperone activity in different environmental conditions (Dimou et al. 2011; Pandey et al. 2016) which are disposing of independent *cis-trans* activity.

In this study, we have characterized Cyclophilin A protein from *Salmonella* that showed high protein peptides sequence homology to CyPs from other organisms and demonstrated the PPIase activity of recombinant protein exhibited by artificial enzyme substrate *in vitro*. Our previous work has reported *cypA* gene cloning and sequence from *Salmonella* Typhimurium showed that the *cypA* gene has high sequence similarity with other virulence bacteria (Manoj et al. 2016; Kumawat et al. 2020). Another study showed that *ppiA*, the gene that is highly conserved among all *Salmonellagenomes*, is a critical role in the growth of *Salmonella* Typhimurium in the examined stress conditions, and may play a role in its responses and virulence (Kumawat et al. 2020). Thus, this study will help to understand the importance of cyclophilin protein in *Salmonella* Typhimurium which may play various biological activities. This will lead to knowing how *Salmonella* regulates virulence factors. Some previous study has confirmed that cyclophilin A protein plays a role in modulating virulence in bacterial species resulting in attenuation *in vivo* (Wang et al. 2001; Roset et al. 2013; Dimou et al. 2017; Bzdyl et al. 2019). However biological functions of *Salmonella* CyPA, their natural substrate and their role in host-parasite relationships are still unclear. PPIases proteins enzyme data suggest that Ppi-A has roles in the protein folding that may be contributing to the virulence of *Salmonella* by isomerization of protein outline.

Peptidyl-prolyl *cis-trans* isomerisation showed more specificity for their protein targets than other chaperones, the purposeful importance of the interplay isn't always continually clean. In this study, we performed a recombinant form of STPpiA protein expression and purified *in vivo*. The proteomic analysis of recombinant form STPpiA proteins showed the significant acceleration of peptidyl-prolyl *cis-trans* isomerisation, a rate-limiting step in protein folding. Possibly these PPIase enable the survival of ST under uncomplimentary conditions. Other PPIases protein activity data suggest that PPIase plays roles in the protein folding that may be contributing to the survival of *Salmonella*. Furthermore, studies are also required to better understand how alterations in protein conformation mediated by PpiA affect cell biology under physiological and stress conditions.

Declarations

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest

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Figures

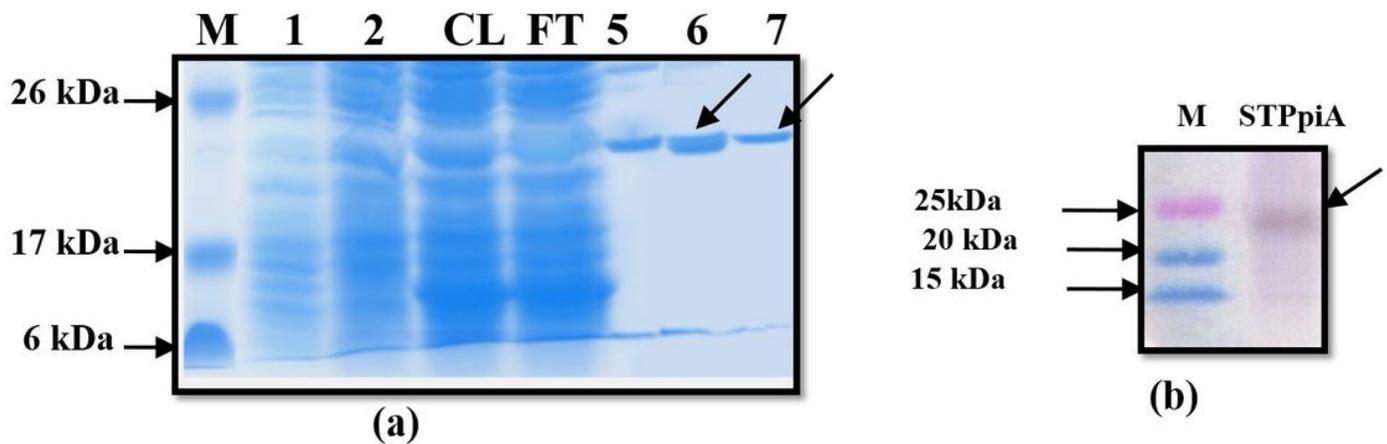


Figure 1

(a) Induction and purification of recombinant PpiA protein expression following:- Lane M is the molecular weight marker (BioRad) Lane 1- uninduced culture, Lane 2- induced culture by IPTG (1mM), Lane CL- column load, Lane FT- flow though, Lane 5-7 are eluted protein fraction. The expressed STPpiA (~24 kDa) protein bands marked by the arrow. (b) Western blot analysis of His-tag purification of STPpiA protein. M is protein ladder markers and recombinant STPpiA band is marked by the arrow.

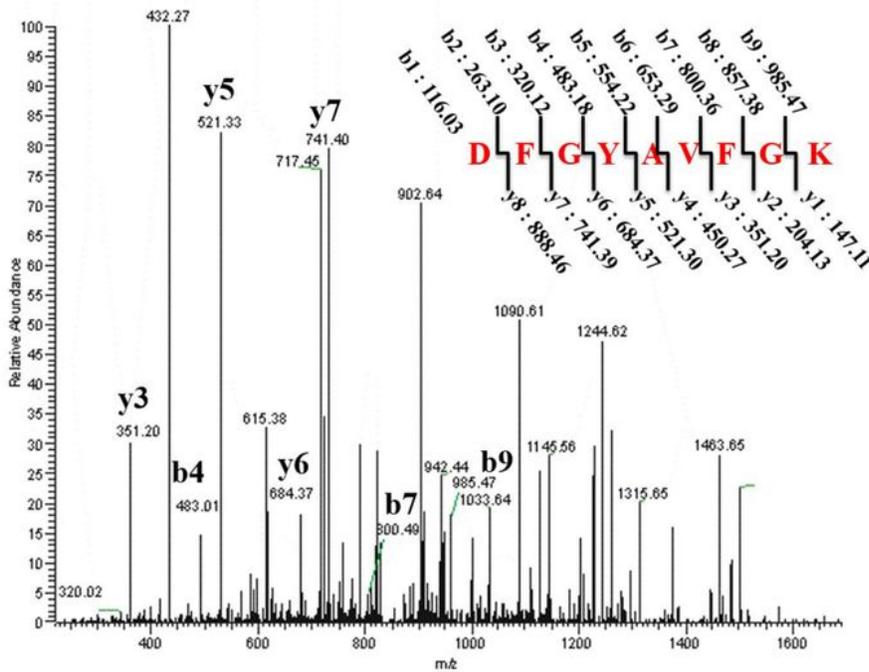
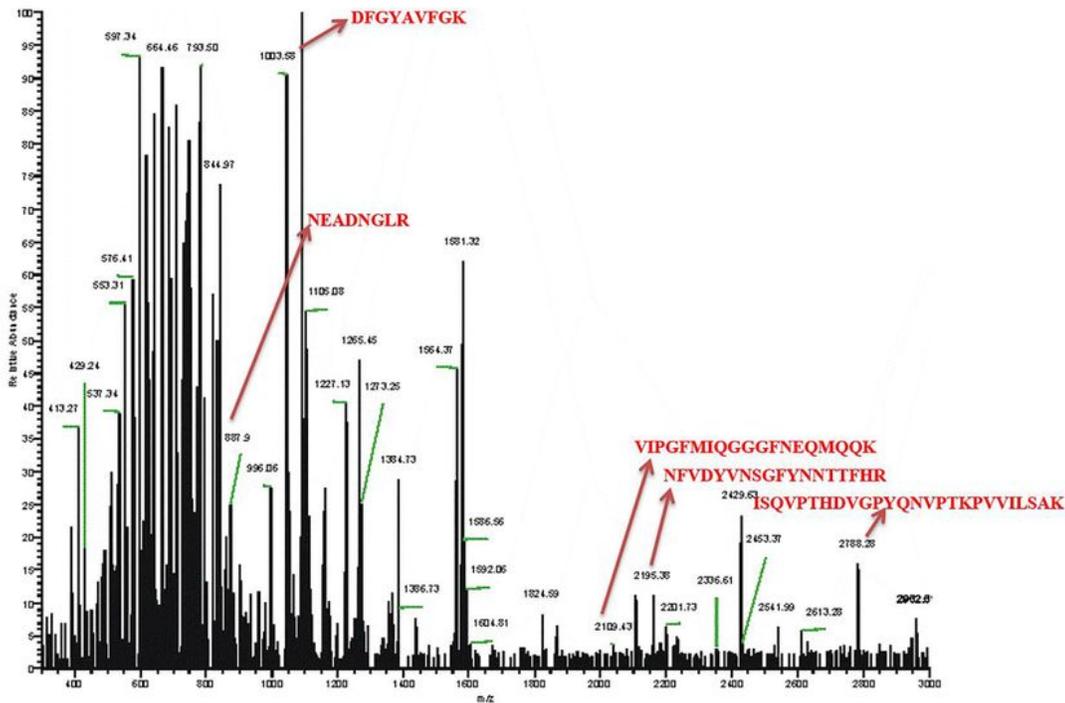


Figure 2

(a) Full LC-mass spectrum (MS) of recombinant STPpiA. The reduced-alkylated STPpiA was digested with trypsin and subjected to LC-MS analysis. Shown here is the mass spectrum acquired mass range 400- 3000. Some m/z values are predicted as protein peptide. (b) LC-MS/MS spectrum of $[M+H]^+$ of DFGYAVFGK (precursor ion m/z 1003.58), residues 143-151 of STPpiA protein. Shown here is the mass spectrum with fragmentation pattern of DFGYAVFGK peptide.

MLKSTLA AVAAVFALSALSPAALAAKGDPHVLLTTSAGNIELELNS
 QKAPVSVK **NFVDYVNSGFYNNTTFHRVIPGFMIQGGGFNEQMQ**
QKKPNPPIKNEADNGLRNTRGTIAMARTADKDSATSQFFINVADN
 AFLDHGQR **DFGYAVFGK**VVKGMDVADK **ISQVPTHVGPYQNV**
TKPVVILSAKVLP

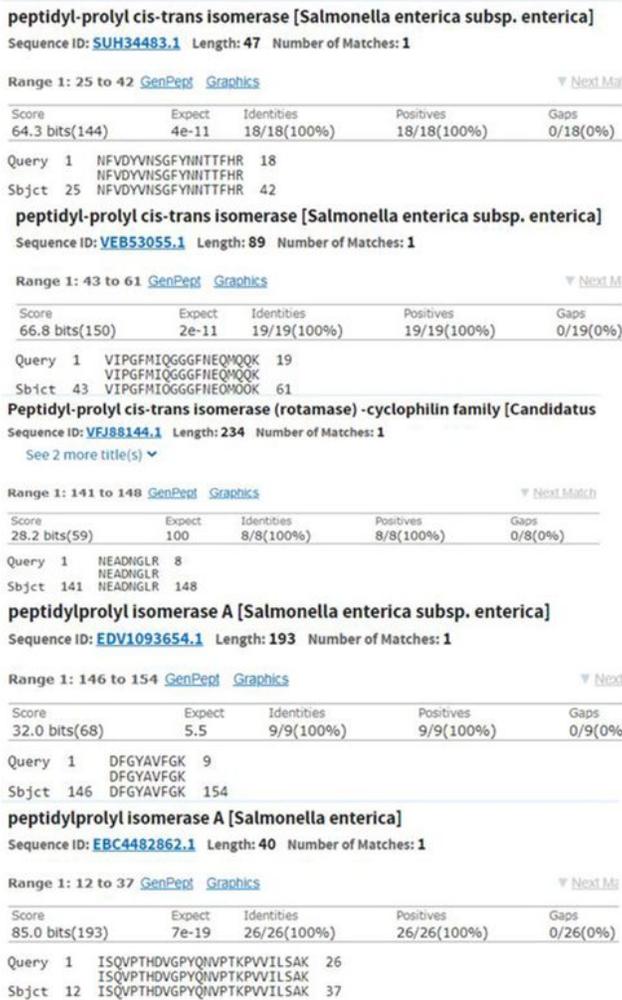


Figure 3

(a) Amino Acid sequence of STPpiA: residues 1-190. The total length of this protein is 163 amino acids, whereas the STPpiA protein covers more than 41 % amino acid residues. (b) Basic local alignment of peptide sequence acquire from MS: Mass spectrometry acquire peptides were subjected for NCBI- BLAST analysis. BLAST result showed higher similarity with PPlases proteins.

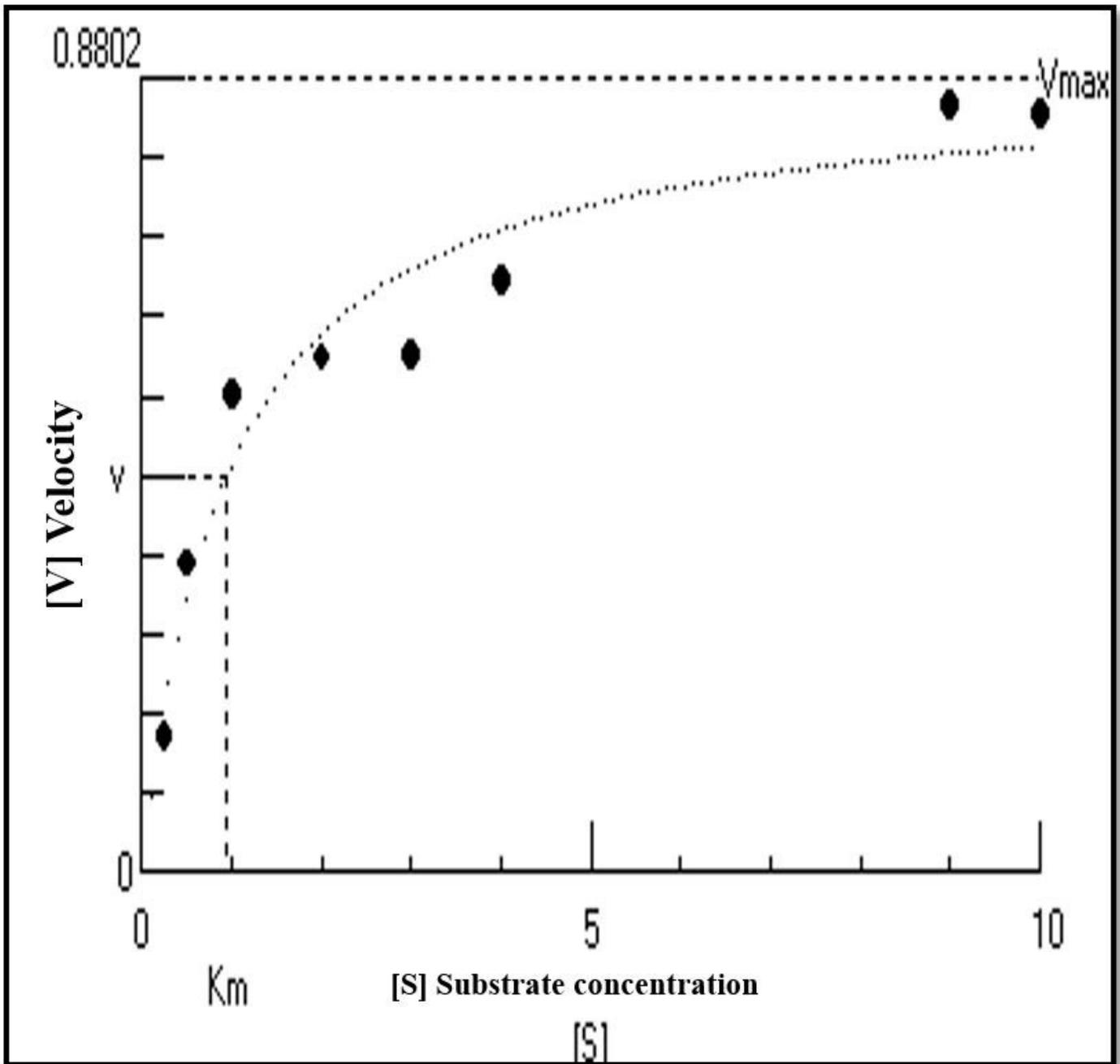


Figure 4

Enzyme kinetics of analysis recombinant purified rPpiA protein. Michaelis-Menten fit based on measured activities of PpiA with the artificial peptide substrates Suc-Ala-Ala-Pro-Phe-pNA. The hyperbolic regression of PpiA protein showed $V_m = 0.8752 \pm 0.13892 \mu\text{moles/ min}$, $K_m = 0.9315 \pm 0.5670 \mu\text{M}$. Data represent average values and standard deviations from duplicate measurements.