Title

Molecular cloning and biochemical characterization of Xaa-Pro dipeptidyl-peptidase from *Streptococcus mutans* and its inhibition by anti-human DPP IV drugs

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Running Title

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Abstract

Streptococcus mutans harbours an intracellular, human DPP IV analogous enzyme Xaa-Pro dipeptidyl-peptidase (EC 3.4.14.11). According to previous reports, an extracellular isozyme in *S. gordonii* and *S. suis* has been associated with virulence. In a speculation that even an intracellular form may aid in virulence of *S. mutans*, we have tried to purify, characterize and evaluate enzyme inhibition by specific inhibitors. The native enzyme was partially purified by ion-exchange and gel filtration chromatography. Owing to low yield, the enzyme was overexpressed in *Lactococcus lactis* and purified by affinity chromatography. The recombinant enzyme (rSm-XPDAP) had a specific activity of 1,070 U/mg, while the V_{max} and K_m were 7 μM min^{-1} and 89 ± 7μM (n = 3), respectively. Serine protease inhibitor phenylmethane-sulphonyl-fluoride and DPP IV specific inhibitor Diprotin A proved to be active against rSm-XPDAP. As a novel approach, the evaluation of anti-human DPP IV (AHD) drugs on rSm-XPDAP activity found saxagliptin to be effective to some extent (K_i = 129 ± 16 μM), which may lead to the synthesis and development of a new class of antimicrobial agents.

Introduction

Streptococcus mutans is one of the major etiological agents of dental caries and may opportunistically cause infective endocarditis (Nakano *et al.* 2010). Genome study as well as proteolytic assays have shown the abundance of peptidases expressed by *S. mutans* (Cowman, Perrella and Fitzgerald 1975; Ajdić *et al.* 2002). Owing to its presence in the oral cavity, these peptidases may facilitate in utilizing salivary polypeptides as a reservoir of nutrition. Aside from nutritional role, proteolysis play an integral role in cell regulation and alleviation of cellular stress, a condition commonly prevailing in the oral cavity (Jenal and Hengge-Aronis 2003). Hence, such proteases can be a suitable target for synthesis of new antimicrobials.

The class of serine proteases includes several bacterial enzymes that endow pathogenic potential. Glutamyl endopeptidase, exfoliative toxin A in *S. aureus*, conserved heat shock protein DegP, IgA1 proteases, trepolisin produced by *Treponema denticola,* tripeptidyl peptidases, prolyl aminopeptidases, serine acyl transferases, Clp proteases are among the serine proteases well studied as possible therapeutic targets (Supuran, Scozzafava and Mastrolorenzo 2001). Xaa-Pro dipeptidyl aminopeptidase (XPDAP) (EC 3.4.14.11) is a narrow range serine protease, which cleaves oligopeptides with a penultimate proline residue from the N-terminus. Pioneering enzymatic studies on XPDAP from *L. lactis* had suggested an orthologous enzyme in *streptococci*, with an implication of this enzyme in pathogenecity (Rigolet *et al.* 2005). In evidence, extracellular XPDAP present in *S. suis* and *S. gordonii* was found to have a role in cellular invasion (Goldstein *et al.* 2001; Ge *et al.* 2009). Other than streptococci, deficiency of XPDAP in periodontal pathogen *Porphyromonas gingivalis* caused altered virulence, through lesser connective tissue destruction and less effective mobilization of inflammatory cells in a mouse abscess model (Yagishita *et al.* 2001). *S. mutans* embodies an intracellular XPDAP (Sm-XPDAP), which shows 50-60% identity

among other oral streptococcal species (Fig. 1). Previous report on the aminopeptidase activity of this bacterium with a more predominating dipeptidyl peptidase activity has emphasized its importance in utilization of proline rich salivary peptides (Cowman and Baron 1993, 1997). Additionally, a collagenolytic and caseinolytic activity may further substantiate the importance of Xaa-Pro dipeptidyl aminopeptidase as a virulence factor and nutritional necessity in *S. mutans*, respectively (Cowman, Perrella and Fitzgerald 1975; Rosengren and Winblad 1976). Overall, these may indicate the potency of Sm-XPDAP as a selective drug target.

Dipeptidyl peptidase IV (DPP IV) (EC 3.4.14.5) is a XPDAP analogous enzyme found in mammals and has been a potent target for maintaining glucose homeostasis in Type II diabetic patients (Wang *et al.* 2012). Certain drugs namely saxagliptin, vildagliptin and sitagliptin are commonly used anti-human DPP IV (AHD) molecules by these patients (Green, Flatt and Bailey 2006). In a speculation that these drug molecules may show an inhibitory effect on *S. mutans* XPDAP (Sm-XPDAP) and thus serve as a template to develop novel molecules specific against this enzyme but not human DPP IV, the gene encoding the enzyme was cloned, purified, characterized and assayed for the inhibition of its activity by DPP IV specific inhibitor and AHD molecules. .

Materials and Methods

Microorganisms and growth conditions

Streptococcus mutans UA159 (ATCC 700610) was used for protein purification and cloning purposes. Glycerol stock of *S. mutans* UA159 was used to grow an overnight culture in Brain Heart Infusion broth (BHI). All the incubations of *S. mutans* cultures were done at 37°C under 5% CO_2 atmosphere (Cowman and Baron 1990).

NICE system (Nisin Controlled gene Expression system, Mobitech) was used for cloning of *pepX* gene from *S. mutans* into *Lactococcus lactis* NZ9000, provided with the kit. It was grown in M17 broth or agar at 30°C supplemented with 0.5% glucose and chloramphenicol (10 μg/mL) as and when required. Electrocompetent cells of *L. lactis* were prepared in M17 broth supplemented with 0.5% glucose, 2.5% glycine and 0.5M sucrose (Holo and Nes 1989).

Partial purification of native Sm-XPDAP

Preparation of cell extracts

A secondary culture (16 L) was prepared and harvested at $O.D_{600}$ of 0.8 (mid-log phase) by centrifugation at 8000×*g* for 15 minutes at 4°C. All the steps were performed at 4°C if not otherwise specified. The cell pellets were washed twice in 10mM phosphate buffer, pH 7.5. The washed cells (25mg), were resuspended in the same buffer for sonication with an ultrasonic homogenizer (Misonix Sonicator 3000), at 12 Watts with each pulse of 30 sec. for 5 times and an intermittent pause of 1 min. The lysate was centrifuged at 8000×g for 15 minutes to remove the cell debris. The supernatant containing the native enzyme was used for further purification of the protein.

Partial purification of native protein

The soluble extract was fractionated with ammonium sulphate, collecting the protein precipitate in the range 50 - 75% saturation by centrifugation at 12,000×g for 20 minutes. The pellet was then gently redissolved in Buffer A (20mM Tris HCl buffer, pH 8.2) and dialyzed against the same buffer overnight. The dialyzed crude extract was bound to pre equilibrated Q-Sepharose column (2.5 x 16 cm), Pharmacia (now GE Healthcare Bio-Sciences AB, Sweden). The proteins were eluted using a linear concentration gradient of NaCl, generated by using 200mL of Buffer A containing 0.55M NaCl with an equal volume of Buffer A at a

flow rate of 0.5mL/min. The active fractions were identified by spectrophotometric analysis at 280nm and by amidolysis in presence of Gly-Pro-*p*-nitroanilide (Gly-Pro-*p*NA). They were then pooled, concentrated by ultrafiltration and dialyzed against Buffer B (20mM Na-K phosphate buffer, pH = 7.4) to be passed through a Superose 12 (GE Healthcare Bio-Sciences AB, Sweden), equilibrated with the same buffer. Proteins were eluted using buffer B containing 0.1M KCl. Similarly, the active fractions from gel filtration were dialyzed against buffer C (20mM Tris HCl, pH = 7.5) and concentrated. A polyanion S1 column (1 x 10 cm) (GE Healthcare Bio-Sciences AB, Sweden), equilibrated with buffer C, was used to separate Sm-XPDAP from the above fraction. The column was eluted using a linear concentration gradient of NaCl (0.5M) in buffer C. The active fractions were dialyzed against buffer A, concentrated and stored at -20 °C until use.

DNA extraction, cloning and protein expression

DNA extraction and cloning

Chromosomal DNA was extracted from an overnight culture of *S. mutans* using a Genome extraction kit (Sigma Aldrich). Primers DPP-F (5'-AATCCATGGCCAAATATAATCAATACAGTTATATCGGTACTTCA-3') and DPP-R (5'-ATAGAGCTCTTAGTGGTGGTGGTGGTGGTGAGCAATGGGGATTTCGATAGTTGA-3') were designed with restriction sites NcoI and SacI in the forward and reverse primers respectively. In order to favour affinity chromatography of the resulting recombinant protein a tandem repeat complementary sequence of 6 Histidine residues (His tag) was incorporated in DPP-R before the SacI site. The amplified fragment containing the gene Sm-*pepX* (SMU _395) and plasmid pNZ8148 (NICE System) were restriction digested using NcoI & SacI at

37°C overnight under appropriate conditions. The digestion products were ligated at 16°C overnight using T4 DNA ligase and electroporated in *L. lactis* NZ9000 using a Gene Pulser (Biorad)

L. lactis electrocompetent cells were prepared as described by Holo and Nes (Holo and Nes 1989). The transformants were confirmed by PCR using extracted fusion plasmids as template DNA and DPP-F and DPP-R as primers. A new set of primers pNZ8148 F-seq (5'-CGGCTCTGATTAAATTCTGAAG-3'), pNZ8148 R-seq (5'-CGTTTCAAGCCTTGGTTTTC-3'), pepX int1 (5'-CCAGTCCCTATCACCAAGGA-3') and pepX int2 (5'-TGCGGAATCTGCTATTTCTTC-3') were used to sequence the insert (service provided by GATC Biotech, Germany).

Protein expression, SDS PAGE and purification by affinity chromatography.

The NisR and NisK regulated protein expression in the bacterial clone was induced by 1 ng/mL Nisin (NICE System - Nisin Controlled gene Expression system, Mobitech) for 3 hours (OD_{600} = 0.4). One millilitre of induced culture pellet was resuspended in reducing dye containing β- mercaptoethanol, boiled for 10 minutes and then centrifuged at 12,000×g for 10 minutes. The supernatant was subjected to a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under standard conditions.

An affinity chromatography mediated by His-tag facilitated the purification of recombinant protein. A batch culture (100 mL) was used to purify recombinant Sm-XPDAP (rSm-XPDAP). The cells were harvested by centrifugation at 8000×g for 15 minutes, washed in 1X Native Purification buffer (NPB) (Invitrogen), followed by resuspension in the same buffer containing 1mg/mL of lysozyme and incubated at 37°C for 30minutes. The protoplast preparation was then disrupted by sonication at 9W for 30 s, 3 pulses each with an interval of 1 minute in ice and the lysate was centrifuged at 8000×g for 15 minutes. A ProBond

Purification system (Invitrogen), under native conditions was used to purify rSm-XPDAP from the crude extract. The active fractions were pooled and analysed by SDS-PAGE. The eluted protein was dialyzed against 0.02M Tris- HCl buffer overnight and stored at -80°C in 20% glycerol. The protein concentration in the enzyme preparations was determined by the Bradford method using bovine serum albumin to set up the standard calibration curve.

Enzyme properties and kinetic studies

Dipeptidyl peptidase IV assays (in-vitro)

The assay was performed in 96 microtiter well plates in 0.02M Tris HCl buffer (pH 7.5) at 37ºC and the amount of p-nitroanilide released was quantified at 410 nm (Yogisha and Ravisha 2010). The K_m and V_{max} values of the purified recombinant Sm-XPDAP (rSm-XPDAP) enzyme were determined in presence of various concentrations of Gly-Pro-*p*NA as the substrate, in the range of 25-400μM.

The effect of pH on the activity of purified recombinant enzyme was checked in the pH range of 4.5 – 9.5. Acetate buffer (pH 4.5 – 5.5), phosphate buffer (pH 5.6 – 7.4), Tris-HCl buffer (pH 7.5 – 8.5) and Glycine NaOH buffer (pH 8.6 – 9.5) were used to compare the enzyme activity. rSm-XPDAP in the presence of respective buffer, pre-equilibrated at room temperature was incubated at 37°C for 5 min after addition of 200 μM substrate.

The temperature dependence of the enzyme activity was determined in Glycine NaOH buffer pH 9.0, in the temperature range 5 - 50°C in presence of 200 μM of substrate. After 5 min incubation the reaction was stopped immediately by addition of 50 μL acetic acid.

The dependence of enzyme activity towards different metal ions were analyzed in presence of 100 μM of Cu^{2+}, Fe^{3+}, Se, Mg^{2+}, Ag, Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+} in 0.02M buffer (pH = 7.5) and 200 μM of substrate. Incubation was achieved at 37°C for 10 min.

In order to evaluate the stability of enzyme at -20°C and -80°C the enzyme was stored for a month, thawed and checked for its activity.

All the readings were taken in triplicates and the basic statistical analyses such as standard deviation, relative standard deviation and unpaired student t-test were performed using MS-Excel workbook.

Effect of antidiabetic drugs and protease inhibitors

The inhibition constants (K_i) of AHD drugs Sitagliptin, Vildagliptin and Saxagliptin (Selleckchem, USA) against rSm-XPDAP were determined using a Dixon plot (Dixon 1953) at various concentrations of drugs in 0.02M buffer (pH 7.5) in two series of substrate concentrations (150µM & 300µM). The concentration of enzyme was 29 mU per assay.

Additionally the effect of some well known protease inhibitors, namely Iodoacetamide, p-chloromercurobenzoate, o-Phenanthroline, Sodium Dodecyl Sulphate (SDS), Phenylmethanesulfonylfluoride (PMSF), EDTA and DPP IV specific inhibitor Diprotin A, was evaluated against rSm-XPDAP.

Results and Discussion

Partial purification of Native Sm-XPDAP

In an attempt to purify Xaa-Pro dipeptidyl-peptidase from *S. mutans*, crude extract was obtained from 16L of batch culture grown till mid log phase. Detection of amidolytic activity against Gly-Pro-*p*NA confirmed the presence of Sm-XPDAP in the crude extract. Initially, batch binding of the enzyme to DEAE-Cellulose and CMC-cellulose failed, which might be due to weak electrostatic interactions of the protein with the resin at pH 7.5. An ammonium sulphate precipitation increased the purity fold of the enzyme, although the yield was drastically affected. In the subsequent steps, the passage of protein through Q-Sepharose,

Superose 12 and Polyanion S1 increased the specific activity of Sm-XPDAP (Table 1). Polyanion S1 increased the specific activity by 17-fold compared to the crude extract. Total protein amounts recovered was very low. As a matter of fact, SDS-PAGE analysis of polyanion S1 active eluate did not show a clear band after Coomassie staining, whereas silver staining showed a band of expected size (Supplementary Figure 1). Nevertheless, the preparation of partially purified native enzyme was used to determine reference biochemical parameters, useful for comparison to over-expressed recombinant Sm-XPDAP.

Cloning and Purification of recombinant enzyme

A 2,276 bp DNA fragment encodes Xaa-Pro dipeptidyl aminopeptidase (XPDAP) in *S. mutans* (Ajdić *et al.* 2002). An initial attempt to clone the gene fragment in an *E. coli* based pET28a system was successful, but the over-expressed protein in the soluble fraction was not active. Hence *Lactococcus lactis* NZ9000 (*pepN*::*nisRnisK*) was used to clone *pepX* (encoding Sm-XPDAP) in the pNZ8148 vector. On Sanger sequencing of the insert, 2 mismatches (His_{106} → Arg_{107} and Ile_{194} → Val_{195}) were found. These mutations are around the N-terminus end of the protein, which should not affect the enzyme activity or proper folding of the active site as judged by 3D computer modeling (data not shown). The sequence identity level of the Sm-XPDAP compared to the other homologous streptococcal enzymes is about 50%. Moreover, a comparative study of the active site of *S. mutans* enzyme with that of other streptococci, bacteria and mammals available in the MEROPS database (Rawlings *et al.* 2014), revealed interesting differences in the Gly-X-Ser-X-X-Gly consensus motif shared by serine proteases. In Sm-XPDAP, there is sequence Gly-Lys-Ser-Tyr-Leu-Gly that maintains the central serine (Ser_{349}), the first and last glycine residues of the consensus motif (Fig. 1, underlined sequence). Lysine (Lys_{348}) is at the second position of the consensus, which is occupied by tryptophan in mammals and Gram-negative bacteria (Ogasawara *et al.* 2005) or by isoleucine or leucine in all other streptococci (Fig. 1). The presence of this amino acid is

noteworthy because a lysine is also found in *Lactococcus lactis* that is the only Gram-positive bacterial species for which the 3D X-ray structure of XPDAP has been resolved (Rigolet *et al.* 2005). This may help in obtaining more useful data from computer modelled Sm-XPDAP structure based on *L. lactis* XPDAP. Tyrosine at position 350 is shared among streptococci and *L. lactis* and is constantly found in the mammalian homologues, while Gram-negative bacteria, such as *Sterotrophomonas maltophilia*, *Pseudomonas* sp. and *Porphyromonas gingivalis*, have asparagine or phenylalanine at the same site (Ogasawara *et al.* 2005). At last Leu_{351} is peculiar of streptococci as it is not found in XPDAP (DPP IV) of other organisms where glycine is constantly present instead. Hence, in streptococci the consensus motif of XPDAP would be Gly-X-Ser-Tyr-Leu-Gly.

A nisin mediated induction of the desired protein was achieved, which showed a distinct protein band of the expected size (Supplementary Figure 2). Moreover, a comparison of the enzyme activity of an induced and uninduced *L. lactis* culture showed a remarkable difference in the rate of substrate hydrolysis ($V_{induced}$ / $V_{uninduced}$ = 17), further confirming that the over-expressed enzyme was active. A batch culture extract was then passed through affinity column. The eluate containing rSm-XPDAP was enzymatically active, pure and showed proper size by SDS-PAGE analysis (86 KDa, Fig.2).

Enzymatic characterization of recombinant Sm-XPDAP

The approximate V_{max} and K_m value of rSm-XPDAP were 7 μM min^{-1} and 89 ± 7μM (n = 3) respectively, the latter being very close to that obtained by the partially purified native enzyme (K_m = 92 μM). rSm-XPDAP was therefore used for the subsequent enzyme characterization study. High enzyme activity may aid in explaining low yield of Sm-XPDAP, which is sufficient to maintain physiological homeostasis in *S. mutans*. The K_m of native enzyme was found lower than both the mammalian enzymes (human : K_m = 0.2 - 0.66 mM;

porcine: K_m = 0.27 mM) (Puschel, Mentlein and Heymann 1982; Caporale *et al.* 1985; Nakajima *et al.* 2008), and the Gram-positive bacterial species XPDAP from *S. anginosus*, *S. suis* and *S. gordonii* studied so far (K_m = 0.56 mM, 0.26 mM and 0.38 mM respectively)(Goldstein *et al.* 2001; Fujimura *et al.* 2005; Jobin *et al.* 2005; Sharoyan *et al.* 2006). It should be noted that lower K_m value suggests higher specificity of Sm-XPDAP towards its substrate. Owing to the higher specificity and ease of purification of Sm-XDAP compared to the mammalian enzyme from tissues, this enzyme may find a potential biotechnological application in food and dairy industry (Prothera and Klaire Labs 2010). On purification, the specific activity of rSm-XPDAP was 1,070 U/mg (1U of enzyme = 1µmole of *p*NA released per minute at 37°C and pH = 7.5).

The optimum pH and temperature of rSm-XPDAP were similar to that of XPDAP in other streptococci (Mineyama and Saito 1991; Jobin *et al.* 2005). Although the optimum intracellular pH of *S. mutans* is 7.0 (Dashper and Reynolds 1992), the optimum pH of the recombinant enzyme was 9.0 (Fig. 3). The enzyme was quite active even at pH 7.5. Considering the intracellular pH of *S. mutans*, the enzyme characteristics were determined at pH 7.5. The optimum temperature lied in the range 30 - 35°C (Fig. 3). Among metal ions, Zn^{2+} showed about 50% inhibition of enzyme activity as also seen in *S. gordonii* XPDAP (Goldstein *et al.* 2001), whereas others had no notable effect on the recombinant enzyme (Table 2).

The serine protease inhibitor phenylmethane-sulphonyl-fluoride (PMSF) and SDS strongly inhibited rSm-XPDAP activity. The sulphydryl group inhibitor iodoacetamide and p-chloro-mercuro- benzoate did not show any enzyme inhibition. EDTA and o-phenanthroline, a metalloprotease inhibitor showed a significant inhibition ($p = 0.0001$ and 0.0034 respectively, $n = 3$, unpaired student t-test, two tailed). This may indicate that Sm-XPDAP is a metalloprotease (Table 2).

The recombinant enzyme did not lose much activity after storage at -80°C for 3 months (74% residual activity). It was quite stable even after repeated freeze and thaw at -20°C (66% residual activity). But a ten times diluted enzyme in Tris buffer was not stable after 24 hours at 4°C.

DPP IV specific inhibition activity against rSm-XPDAP

Like all other bacterial DPP IVs, DPP IV specific tripeptide Diprotin A was used as a reference inhibitor that showed a K_i of 16.7 µM. In view of the important role of Sm-XPDAP in *S. mutans* metabolism and its homology with the human enzyme, the effect of antidiabetic drugs on rSm-XPDAP was determined. Although sitagliptin and vildagliptin did not show any inhibition at 100 µM, saxagliptin was to some extent active and competitively inhibited rSm-XPDAP activity. An inhibition constant (K_i) of 129 ± 16 µM was determined using a Dixon plot (Dixon 1953). The results were consistent with that observed in case of human DPP IV, where saxagliptin is most effective due to its strong interaction with two amino acids Ser_{630} and $Glu_{205/206}$ compared to Ser_{630} in case of vildagliptin (Wang *et al.* 2012). Similar interactions of saxagliptin at Ser_{349} and $Glu_{393/396}$ of Sm-XPDAP may result in its higher inhibition. Inhibition of enzyme activity at 100µM of saxagliptin was significantly different from that of 50µM of the drug ($p = 0.037$, paired t-test). Vildagliptin inhibited rSm-XPDAP by 47% at 900 µM, while 1mM of sitagliptin could exhibit only 37% enzyme inhibition (Table 3). At 500µM, both vildagliptin and sitagliptin showed slight inhibition of enzyme activity, although vildagliptin was more potent than the other ($p<0.01$, unpaired student t-test). Anticipating Sm-XPDAP as a potent antibacterial target, high inhibition constant (K_i) values of saxagliptin in an *in vitro* condition rules out to evaluate its antimicrobial efficacy, assuming that the MIC_{50} will be high. This is a good indication as this drug is designed to act against human DPP IV. But, this may serve as a lead compound towards development of

molecules with lower inhibition constant against Sm-XPDAP and consequently as a potent antimicrobial. In addition, a higher effect by saxagliptin and vildagliptin may suggest that cyanopyrrolidide group can be a good starting scaffold to synthesize new molecules.

In conclusion, Sm-XPDAP, an intracellular endopeptidase, is analogous to human DPP IV and is inhibited by antihuman DPP IV drug saxagliptin. Comparative study of XPDAP sequences revealed a unique sequence identity of the Sm-XPDAP in the consensus motif. These can lead to anticipate Sm-XPDAP as a novel antimicrobial target and consequently develop a new approach to treat caries. Future studies will be focusing on construction of Sm-XPDAP knock out strain and development of new compounds inhibiting Sm-XPDAP.

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Conflicts of interests

Authors have no conflict of interest to declare.

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Figure legends

Figure 1. Sequence Alignment of Xaa-Pro dipeptidyl aminopeptidase of various oral bacteria. Sequences of various oral streptococci (*S. mutans* (MER022661), *S. salivarius* (MER188504), *S. oralis (*MER360252), *S. mitis* (MER360250), *S. sanguinis* (MER299432), *S. gordonii* (MER015080), *S. pneumonia* (MER014989), *S. thermophilus* (MER026289), *S. suis* (MER043202)), and *Lactococcus lactis* (MER014202) obtained from MEROPS and aligned using the program CLUSTALW (Thompson, Higgins and Gibson 1994). The serine protease consensus sequence is underlined, serine of the catalytic site is indicated by an asterisk and the residues mutated in the recombinant protein by empty circles.

Figure 2. SDS-PAGE analysis of active protein fraction after affinity chromatography. Lane 1: Molecular marker, Lane 2: Purified rSm-XPDAP.

Figure 3. Effect of pH and temperature on rSm-XPDAP activity. The plots represent mean ± SD of percentage residual activity from three independent experiments.