Supplementary material

Fermentation

Genetic and Biochemical Characterization of a Novel Alkaline-tolerant Xaa-Pro Dipeptidase from *Aspergillus phoenicis*

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Table S1. Xaa-Pro dipeptidases that have been characterized by molecular and/or biochemical techniques

Enzyme	Organism	GenBank accession No.	Optimum pH and temperature	pH stability and thermostability	Monomer/Multimer (MW of subunits, or native enzymes) [a]	Inhibitor (cofactor) [b]	Substrate specificity [c]	Reference
Hyperthermophilic archaea								
PfPEPQ	*Pyrococcus furiosus* DSM 3638	P81535.1	pH 7.0, 100ºC	Half life at 100ºC for native and recombinant enzymes is 4 h and 1 h, respectively	Homodimer (39.4 kDa, 100±10 kDa)	EDTA, Zn^{2+} (Co^{2+}, Mn^{2+})	Xaa-Pro (Xaa=Met, Leu, Val, Phe or Ala)	[1-3]
PhPROL	*P. horikoshii* OT3	BAA30249.1	pH 7.0, 100ºC	Half life at 90ºC is 21.5 h	39.27 kDa, NA	Zn^{2+} (Co^{2+})	Met-Pro, Leu-Pro, and organophosphorus nerve agents	[4,5]
Eubacteria								
AhOPAA	*Alteromonas haloplanktis* ATCC	P77814.1	pH 7.5, 40ºC		Monomer (50 kDa, NA)	DFP analog Nipafox, pCMB,	Xaa-Pro and OP compounds	[6]

	23821					NEM (Mn^{2+})		
AmOPAA	*A. macleodii* NCIMN1963	AEI26268.1			Dimer (50.6 kDa, 101.2 kDa)	cofactor: Mn^{2+}	Xaa-Pro and OP compounds	[7]
AsOPAA	*Alteromonas* sp. JD6.5	Q44238.3	pH 8.5, 50ºC	ND [a]	Monomer (60 kDa, 60 kDa)	pCMB, IAA, NEM, EGTA, Zn^{2+} (Mn^{2+})	DFP, NPMPP, NPEPP, paraoxon and nerve agents such as sarin, soman, and O-cyclohexyl methylphosphonofluoridate; Xaa-Pro dipeptides such as Gly-Pro	[8-10]
AuOPAA	*A. undina* ATCC 29660		pH 8.0, 55ºC		Monomer (53 kDa, 53 kDa)	IAA, EGTA, NEM, Ni^{2+} and Zn^{2+} (Mn^{2+})	A wide range of nerve agents and several chromogenic phosphinates	[11]
DrXPD	*Deinococcus radiodurans* R1	WP_027479553.1			Dimer (45 kDa, NA)	cofactor: Mn^{2+}	Xaa-Pro	[12]
EcPEPQ	*Escherichia coli* BL21 (DE3)	P21165.2	pH 8.0, 60ºC	Incubation at 4-30ºC for 10 min (>80%), incubation at pH 7.0-9.0 for 1 h	Dimer	cofactor: Mn^{2+}	Xaa-Pro dipeptides, DFP, organophosphate diesters and triesters, and nerve agents GB (sarin), GD	[13-15]

				(30°C, >60%)			(soman), GF and VX	
LcXPD	*Lactobacillus casei* subsp. *casei* IFPL 731		pH 6.5-7.5, 55°C	Retain 36% activity after incubation at 30°C for 30 min	Monomer (41 kDa, 41 kDa)	EDTA and 1,10-phenanthroline, DTT, ME, pHMB and IAA , Cu^{2+}, Zn^{2+} , Fe^{3+} (Mn^{2+}, Co^{2+})	Hydrolyze Xaa-Pro, Ala-Ala and Ala-Phe except Pro-Pro and Gly-Pro	[16]
LdPEPQ	*L. delbrueckii* subsp. *bulgaricus* CNRZ 397	CAA73815.1	pH 6.0, 50°C		Homodimer (45 kDa, 68-70 kDa)	Bestatin, DTT, EDTA (Zn^{2+})	Hydrolyzes Xaa-Pro dipeptides except for Pro-Pro and Gly-Pro	[17]
LlXPDI	*Lactococcus lactis* subsp. *cremoris* AM2		8.3-9.0 (universal buffer)		NA, 42 kDa	NEM, benzamidine, bestatin, bacitracin, PMSF, pCMB, IAA, leupeptin	Xaa-Pro, Pro-Pro, Pro-Ala, Pro-Val, etc.	[18]
LlXPDII	*L. lactis* NRRL B-1821	ABW84230.1	pH 7.0, 40-50°C	Incubation at 20-50°C for 30 min (>60%)	Dimer (40 kDa, 80 kDa)	cofactor: Zn^{2+}	Leu-Pro, Arg-Pro, Val-Pro, Phe-Pro and Lys-Pro; doesn't hydrolyze Gly-Pro, Glu-Pro, Asp-Pro, Pro-Pro,	[19]

							Leu-Leu-Pro and Leu-Val-Pro	
MtXPD	*Mycobacterium tuberculosis* H37Rv	P9WHS7.1	pH 7.5-8.0, 55°C	Completely loss its activity at 50°C and 65°C in the absence and presence of 1 mM Mn^{2+}, respectively		Phosphate ion, cacodylate	Hydrolyze only dipeptides, but failed to cleave tripeptides or long chain peptide like bradykinin	[20]
PlOPAA	*Pseudoalteromonas lipolytica* SCSIO04301	WP_036973676	pH 8.5, 55°C	Incubation at pH 5.0-8.0 for 2 h (>60%); half-lives at 55, 60 and 65°C are 88.87, 78.77 and 33.49 min, respectively	Tetramer (53 kDa, 200 kDa)	EDTA, Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+} (Mn^{2+})	Gly-Pro, shows preference for oxon-phosphoryl than thiono-phosphoryl, hydrolyzes dichlorvos, methyl-paraoxon, paraoxon and profenofos	[21,22]
ScXPD	*Streptococcus cremoris* H61		pH 6.5-7.5, 40°C	Quickly lose activity at temperatures above 40°C	Monomer (NA, 43 kDa)	EDTA and 1,10-phenanthroline, pCMB, NEM, ME, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}	Xaa-Pro, e.g. Leu-Pro	[23]

						(Co^{2+})		
TsPROL	*Thermococcus sibiricus* MM 739	ACS89882.1			Homodimer (39 kDa, 70-75 kDa)	cofactor: Zn^{2+}		[24,25]
XcXPD	*Xanthomonas campestris* pv. *campestris* ATCC 33913	Q8P839	pH 7.5-8.0, 55°C	Completely loss its activity at 60°C and 80°C in the absence and presence of 1 mM Mn^{2+}, respectively	Dimer	Phosphate ion, cacodylate (Mn^{2+})	Hydrolyze only dipeptides, but failed to cleave tripeptides or long chain peptide like bradykinin	[20,26]
XmXPD	*X. maltophilia*		pH 7.5, 35°C	pH 6.0-8.5; half life at 37°C is 60 min	Dimer (51 kDa, 100 kDa)	pCMB, o-phenanthroline, Z-L-proline, and phenylacetyl-thio proline, Cu^{2+}, Zn^{2+}, Hg^{2+} (Mn^{2+})	Xaa-Pro, e.g. Leu-Pro	[27]
				Eukarya				
AnPEPP	*Aspergillus*	CAC39600.1	pH 7.0	Stable at 40°C for 60	Homodimer (58	cofactor: Mn^{2+}	Met-Pro, Ala-Pro, Phe-Pro, Leu-Pro and Val-Pro; could	[28]

	nidulans WG312			min	kDa, 125 kDa)		not hydrolyze Pro-Ala, Ala-Ala and Ala-Pro-Gly	
HsPEPDI	*Homo sapiens*	AAA60064.1	pH 7.8, 50°C	37°C for at least 8 days	Dimer (54 kDa, NA) [c]	Cbz-Pro, guanidine hydrochloride (Mn^{2+})	Gly-Pro>Ala-Pro>Phe-Pro>Leu-Pro, DFP, sarin, soman, tabun and cyclosarin	[29-31]

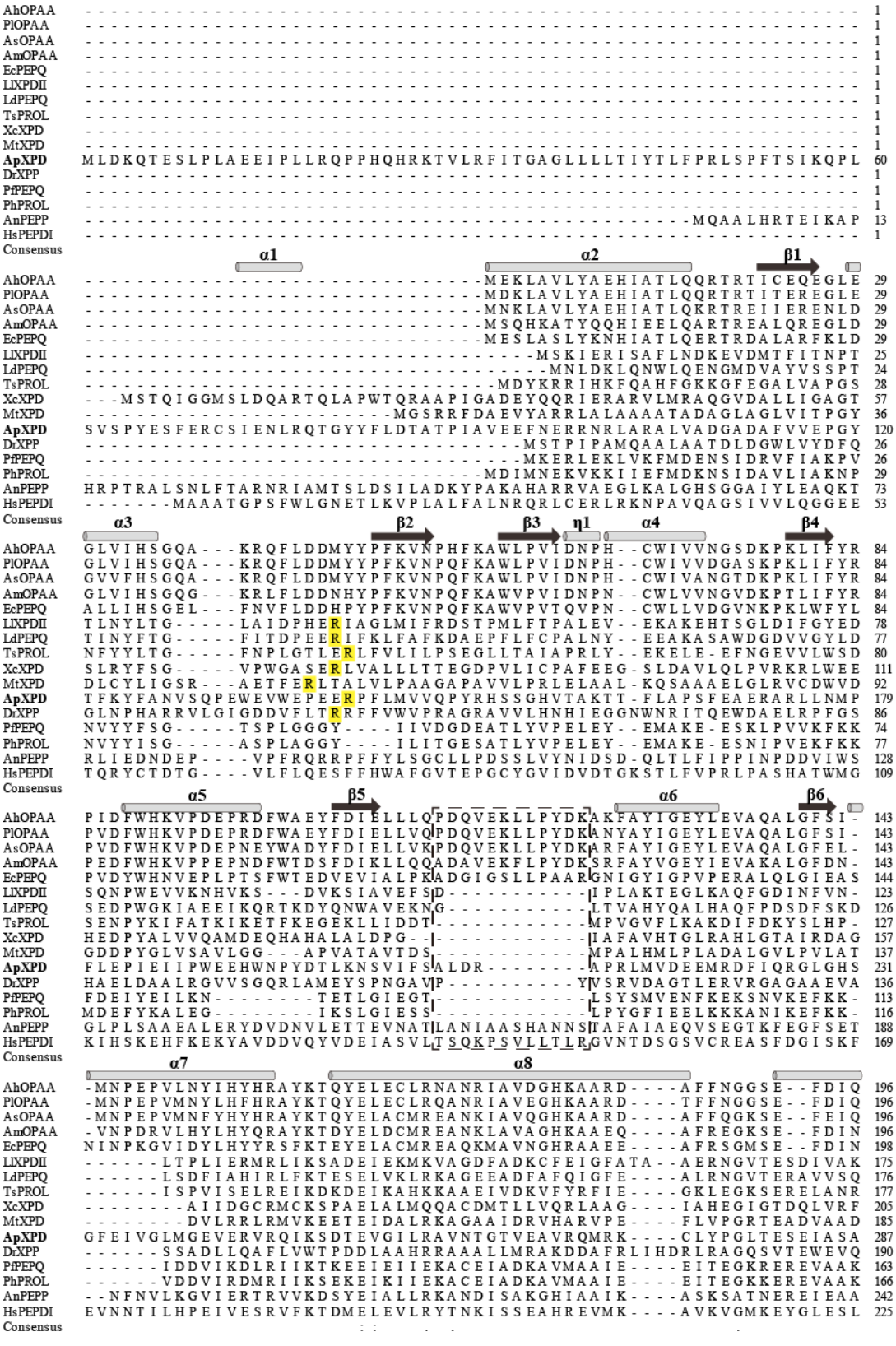
ND, not determined; NA, not available;

[a] Molecular weights of subunits and native enzymes were determined by SDS-PAGE and gel filtration, respectively;

[b] pCMB, *p*-chloromercuribenzoic acid; IAA, iodoacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; pHMB, *p*-hydrocymercuribenzoic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; ME, *β*-mercaptoethanol

[c] DFP, diisopropylfluorophosphate; NPMPP, *p*-nitrophenylmethyl(phenyl)phosphinate; NPEPP, *p*-nitrophenylethyl(phenyl)phosphinate; GF, *O*-cyclohexylmethylphosphonofluoridate; VX, *S*-2(diisopropylamino)ethyl *O*-ethyl methylphosphonothioate; OP compounds, organophosphate compounds.

Figure S1. Multiple sequence alignment of ApXPD with all the characterized XPDs from various organisms. (*), the conserved amino acids; (:), the conservative replacement; (.), semiconservative replacement. Dashes indicate gaps to maximize alignment. The secondary structural elements of XcXPD are shown above the sequence. The strictly conserved motifs HXXGHXXGXXXH and GXRXE (X represents any amino acid) are boxed. Four regions with at least ten residues that appear in prolidases from subfamilies M24.003 and M24.007 but are absent in members of the unknown and M24.008 subfamilies are indicated by dashed rectangles. The putative catalytic triads, metal ion binding residues and the specific arginine residues that are mainly determining the substrate length are highlighted in purple, green and yellow, respectively. Ten additional residues in ApXPD are shown in red.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α3
β2
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α4
β4
α5
β5
α6
β6
α7
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PlOPAA
AsOPAA
AmOPAA
EcPEPQ
LlXPDII
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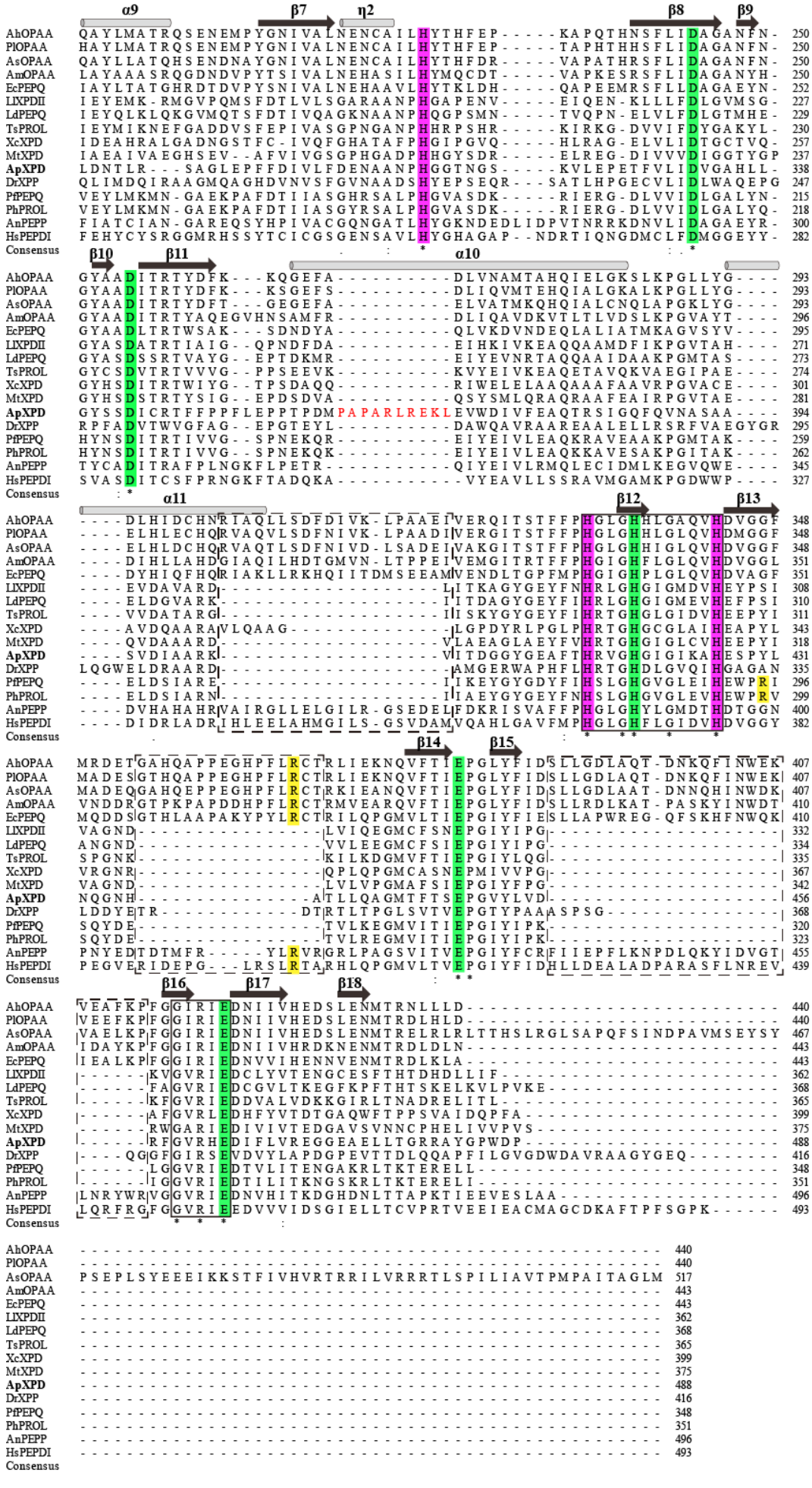
Consensus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LlXPDII
LdPEPQ
TsPROL
XcXPD
MtXPD
ApXPD
DrXPP
PfPEPQ
PhPROL
AnPEPP
HsPEPDI
Consensus

References

1. Ghosh, M., .; Grunden, A.M.; Dunn, D.M.; Weiss, R., .; Adams, M.W. Characterization of native and recombinant forms of an unusual cobalt-dependent proline dipeptidase (prolidase) from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* **1998**, *180*, 4781-4789.

2. Grunden, A.; Ghosh, M.; Schut, G. Proline dipeptidase from *Pyrococcus furiosus*. *Methods Enzymol.* **2001**, *330*, 433-445.

3. Du, X.L.; Tove, S.; Kast-Hutcheson, K.; Grunden, A.M. Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase by analysis of targeted mutants. *FEBS Lett.* **2005**, *579*, 6140-6146.

4. Theriot, C.M.; Tove, S.R.; Grunden, A.M. Characterization of two proline dipeptidases (prolidases) from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 177-188.

5. Theriot, C.M.; Semcer, R.L.; Shah, S.S.; Grunden, A.M. Improving the catalytic activity of hyperthermophilic *Pyrococcus horikoshii* prolidase for detoxification of organophosphorus nerve agents over a broad range of temperatures. *Archaea* **2011**, *2011*, 565127.

6. Cheng, T.; Liu, L.; Wang, B.; Wu, J.; DeFrank, J.J.; Anderson, D.M.; Rastogi, V.K.; Hamilton, A.B. Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from *Alteromonas haloplanktis*. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 49-55.

7. Stepankova, A.; Duskova, J.; Skalova, T.; Hasek, J.; Koval, T.; Ostergaard, L.H.; Dohnalek, J. Organophosphorus acid anhydrolase from *Alteromonas macleodii*: structural study and functional relationship to prolidases. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2013**, *69*, 346-354.

8. DeFrank, J.J.; Cheng, T.C. Purification and properties of an organophosphorus acid anhydrase from a halophilic bacterial isolate. *J. Bacteriol.* **1991**, *173*, 1938-1943.

9. Cheng, T.C.; Harvey, S.P.; Chen, G.L. Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. *Appl. Environ. Microbiol.* **1996**, *62*, 1636-1641.

10. Vyas, N.K.; Nickitenko, A.; Rastogi, V.K.; Shah, S.S.; Quiocho, F.A. Structural insights into the dual activities of the nerve agent degrading organophosphate anhydrolase/prolidase. *Biochemistry* **2010**, *49*, 547-559.

11. Cheng, T.C.; Harvey, S.P.; Stroup, A.N. Purification and properties of a highly active organophosphorus acid anhydrolase from *Alteromonas undina*. *Appl. Environ. Microbiol.* **1993**, *59*, 3138-3140.

12. Are, V.N.; Jamdar, S.N.; Ghosh, B.; Goyal, V.D.; Kumar, A.; Neema, S.; Gadre, R.; Makde, R.D. Crystal structure of a novel prolidase from *Deinococcus radiodurans* identifies new subfamily of bacterial prolidases. *Proteins* **2017**, *85*, 2239-2251.

13. Park, M.S.; Hill, C.M.; Li, Y.; Hardy, R.K.; Khanna, H.; Khang, Y.H.; Raushel, F.M. Catalytic properties of the PepQ prolidase from *Escherichia coli*. *Arch. Biochem. Biophys.* **2004**, *429*, 224-230.

14. Weaver, J.; Watts, T.; Li, P.; Rye, H.S. Structural basis of substrate selectivity of *E. coli* prolidase. *PLoS One* **2014**, *9*, e111531.

15. Wang, T.F.; Chi, M.C.; Lai, K.L.; Lin, M.G.; Chen, Y.Y.; Lo, H.F.; Lin, L.L. High-level expression and molecular characterization of a recombinant prolidase from *Escherichia coli* NovaBlue. *PeerJ* **2018**, *6*, e5863.

16. Fernandez-Espla, M.D.; Martin-Hernandez, M.C.; Fox, P.F. Purification and characterization of a prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731. *Appl. Environ. Microbiol.* **1997**, *63*, 314-316.

17. Morel, F.; Frot-Coutaz, J.; Aubel, D.; Portalier, R.; Atlan, D. Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis. *Microbiology* **1999**, *145 (Pt 2)*, 437.

18. Booth, M.; Jennings, V.; Fhaolain, I.N.; O'Cuinn, G. Prolidase activity of *Lactococcus lactis* subsp. *cremoris* AM2: partial purification and characterization. *J. Dairy Res.* **1990**, *57*, 245-254.

19. Yang, S.I.; Tanaka, T. Characterization of recombinant prolidase from *Lactococcus lactis*- changes in substrate specificity by metal cations, and allosteric behavior of the peptidase. *FEBS J.* **2008**, *275*, 271-280.

20. Are, V.N.; Kumar, A.; Kumar, S.; Goyal, V.D.; Ghosh, B.; Bhatnagar, D.; Jamdar, S.N.; Makde, R.D. Crystal structure and biochemical investigations reveal novel mode of substrate selectivity and illuminate substrate inhibition and allostericity in a subfamily of Xaa-Pro dipeptidases. *BBA-Proteins Proteom.* **2017**, *1865*, 153-164.

21. Xiao, Y.Z.; Yang, J.; Tian, X.P.; Wang, X.X.; Li, J.; Zhang, S.; Long, L.J. Biochemical basis for hydrolysis of organophosphorus by a marine bacterial prolidase. *Process Biochem.* **2017**, *52*, 141-148.

22. Yang, J.; Xiao, Y.; Liu, Y.; Li, R.; Long, L. Structure-based redesign of the bacterial prolidase active-site pocket for efficient enhancement of methyl-parathion hydrolysis. *Catal. Sci. Technol.* **2021**, *11*, 5086-5093.

23. Kaminogawa, S.; Azuma, N.; Hwang, I.K.; Suzuki, Y.; Yamauchi, K. Isolation and characterization of a prolidase from *Streptococcus cremoris* H61. *J. Agr. Chem. Soc. JPN.* **1984**, *48*, 3035-3040.

24. Timofeev, V.; Slutskaya, E.; Gorbacheva, M.; Boyko, K.; Rakitina, T.; Korzhenevskiy, D.; Lipkin, A.; Popov, V. Structure of recombinant prolidase from *Thermococcus sibiricus* in space group P21221. *Acta Crystallogr.* **2015**, *71*, 951-957.

25. Trofimov, A.A.; Slutskaya, E.A.; Polyakov, K.M.; Dorovatovskii, P.V.; Gumerov, V.M.; Popov, V.O. Influence of intermolecular contacts on the structure of recombinant prolidase from *Thermococcus sibiricus*. *Acta Crystallogr.* **2012**, *68*, 1275-1278.

26. Kumar, A.; Are, V.N.; Ghosh, B.; Agrawal, U.; Jamdar, S.N.; Makde, R.D.; Sharma, S.M. Crystallization and preliminary X-ray diffraction analysis of Xaa-Pro dipeptidase from *Xanthomonas campestris*. *Acta Crystallogr. F Struct. Biol. Commun.* **2014**, *70*, 1268-1271.

27. Suga, K.; Kabashima, T.; Ito, K.; Tsuru, D.; Okamura, H.; Kataoka, J.; Yoshimoto, T. Prolidase from *Xanthomonas maltophilia*: purification and characterization of the enzyme. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 2087-2090.

28. Jalving, R.; Bron, P.; Kester, H.C.; Visser, J.; Schaap, P.J. Cloning of a prolidase gene from *Aspergillus nidulans* and characterisation of its product. *Mol. Genet. Genomics* **2002**, *267*, 218-222.

29. Lupi, A.; Della Torre, S.; Campari, E.; Tenni, R.; Cetta, G.; Rossi, A.; Forlino, A. Human recombinant prolidase from eukaryotic and prokaryotic sources. Expression, purification, characterization and long-term stability studies. *FEBS J.* **2006**, *273*, 5466-5478.

30. Chandrasekaran, L.; Belinskaya, T.; Saxena, A. *In vitro* characterization of organophosphorus compound hydrolysis by native and recombinant human prolidase. *Toxicol. in Vitro.* **2013**, *27*, 499-506.

31. Besio, R.; Baratto, M.C.; Gioia, R.; Monzani, E.; Nicolis, S.; Cucca, L.; Profumo, A.; Casella, L.; Basosi, R.; Tenni, R.; et al. A Mn(II)–Mn(II) center in human prolidase. *BBA - Proteins and Proteom.* **2013**, *1834*, 197-204.