

Cloning and sequencing of protein *L*-isoaspartyl *O*-methyl transferase of *Salmonella* Typhimurium isolated from poultry

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Abstract

Aim: To clone the *Salmonella* Typhimurium protein *L*-isoaspartyl *O*-methyl transferase (*PIMT*) enzyme and to analyze the sequence with *PIMT* gene of other pathogenic serovars of *Salmonella*.

Materials and Methods: *Salmonella* Typhimurium strain E-2375 was procured from the National Salmonella Center, IVRI. The genomic DNA was isolated from *Salmonella* Typhimurium. Polymerase chain reaction (PCR) was carried out to amplify *PIMT* gene using the designed primers. The PCR product was cloned into pET28c plasmid vector and transformed into *Escherichia coli* DH5a cells. The plasmid was isolated from *E. coli* and was sequenced. The sequence was analyzed and submitted in Genbank.

Results: The PCR product revealed a distinct amplicon of 627 bp. The clone was confirmed by PCR. Sequencing data revealed 100% homology between *PIMT* sequences from *Salmonella* Typhimurium strain E-2375 (used in the current study) and *PIMT* sequences of standard reported strain (*Salmonella* Typhimurium str. LT2) in NCBI data base. This submitted sequence in Genbank having accession no. KJ575536.

Conclusions: *PIMT* gene of *Salmonella* is highly conserved in most of the pathogenic *Salmonella* serovars. The *PIMT* clone can be used to isolate *PIMT* protein. This *PIMT* protein will be helpful to identify isoaspartate containing proteins thus can help in study *Salmonella* virulence.

Keywords: cloning, sequencing, *Salmonella* Typhimurium protein *L*-isoaspartyl *O*-methyl transferase, virulence.

Introduction

Salmonella enterica serovar Typhimurium (shortly *Salmonella* Typhimurium) is a Gram-negative, flagellated and facultative intracellular anaerobe grouped under the family enterobacteriaceae is widely known for its highly zoonotic importance [1]. This pathogenic bacterium infects wide range of animal host causing severe morbidity and mortality in animals and human [2,3]. Poultry birds mainly act as an asymptomatic reservoir host and its contaminated meat and eggs are the source of infection causing severe gastroenteritis in human and typhoid like disease in rodents [4]. Growing resistance to antibiotics [5], endemicity of infection and their career state encourages understanding the several aspects of its physiology and metabolism for novel vaccine development and its effective control. There are several virulence factor associated with the severity of infection [6]. The virulence associated enzyme mediated protein repairing modification mechanism associated with *Salmonella* for the confrontation of a hostile environment inside the host immune system. The phagocytic cells damage various macromolecules such as DNA,

RNA and proteins by producing a battery of antimicrobials such as reactive oxygen species and reactive nitrogen species [7]. The proteins are the major target for this oxidative damage due to their abundance and oxidation leads to covalent modification of the amino acid residues and conformational changes in protein structure [8]. This protein modification brought about by various protein repairing enzymes may be present in *Salmonella* Typhimurium rescue from the oxidative damage and assist for intraphagosomal survival and its replication.

Out of the several protein repairing enzymes reported, protein *L*-isoaspartyl *O*-methyl transferase (*PIMT*) have been found to be effective for all the domains of the life, and this enzymes extensively studied in *Escherichia coli* [9] and *Pyrococcus* [10] and *Vibrio cholera* [11]. The way *PIMT* works are by repairing the damaged proteins like *L*-isoaspartyl residues to normal *L*-aspartyl in proteins by methyl transferase activities [12]. The housekeeping role of this enzyme catalyzes the methyl esterification by transfer of methyl group from the *S*-adenosylmethionine to the α -carboxyl group of *L*-isoaspartyl and β -carboxyl group of *D*-aspartyl residues via succinimide intermediate formation, which is hydrolyzed to a mixture of *L*-aspartyl and *L*-isoaspartyl residues in the ratio of 1:3 and subsequently restore their biological functions [13].

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As per our knowledge, no work has been reported until date regarding the importance of *PIMT* in *Salmonella* Typhimurium. Keeping this in view, the present study was carried out to clone and sequence the *PIMT* gene of *S. Typhimurium* and to analyze the sequence relationship with other pathogenic *Salmonella*.

Materials and Methods

The poultry isolate of *Salmonella* Typhimurium strain E-2375 was procured from National *Salmonella* Centre (Veterinary), Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, India. The cultures were revived by growing in LB broth at 37°C overnight, and the isolated colonies were obtained by streaking on Hektoen Enteric Agar (HEA) plate. The cultures were confirmed by testing the purity, morphology and biochemical characteristics as per standard protocol [14]. Serotype confirmation was done by slide agglutination test using polyvalent antisera and tube agglutination test using somatic and flagellar group specific and factor antisera.

The genomic DNA was isolated as per the standard protocol (purelink™ genomic DNA isolation kit, Cat. no. K-1820-01, Invitrogen™, USA). To check the integrity of the DNA, about 0.5 µg sample was run on 1% w/v agarose gel (Ultrapure™ agarose, Invitrogen, USA) prepared in 1X TAE [15]. The *PIMT* of *Salmonella* Typhimurium was amplified by using designed oligonucleotides primers (Table-1). The restriction enzymes *NheI* and *BamHI* sites were incorporated on 5' of forward and reverse primers, respectively.

PCR reaction was carried out using following constituents, 7.5 µl of 2 mM dNTPs mix, 1.5 µl of *PIMT* FP and *PIMT* RP (10 pM/µl each), 1 µl of 50 mM MgCl₂, 5 µl ×10 PCR reaction buffer, 5 µl Enhancer, 2 µl (96 ng/µl) of *Salmonella* Typhimurium genomic DNA as template and 0.5 µl (2.5 U/µl) of *Pfx* polymerase and the final reaction volume made up to 50 µl with Nuclease free water. The PCR program was standardized with one cycle of the initial denaturation at 95°C for 5 min, followed by 30 cycles each of denaturation (95°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 45 s) and final extension (72°C for 10 min). The PCR products were checked by submarine gel electrophoresis on 1% agarose gel.

The amplified PCR product digested with *NheI* (5 h at 37°C) and then, *BamHI* was added to

the reaction, and the mix was incubated for an additional 1 h. Finally, heat inactivation was carried out at 80°C for 10 min. The RE digested *PIMT* PCR product was gel purified using QIA quick gel extraction kit (Qiagen™, Germany) using manufacturers protocol. The pET28c vector was digested in a similar manner with *NheI* and *BamHI* restriction enzymes to generate complementary overhangs. Following RE digestion the vector was dephosphorylated at 37°C for 30 min using shrimp alkaline phosphatase. The enzymes were heat inactivated at 80°C for 10 min. The digested and dephosphorylated pET28c was gel purified. *NheI*-*BamHI* digested, gel purified *PIMT* gene was ligated into the digested pET28c expression vector. A 3:1 molar ratio of insert (*PIMT*) to vector (pET28c) was used. The resultant recombinant plasmid (pET28c-*PIMT* Plasmid) was initially transformed into chemically competent *E. coli* DH5α cells with 5 µl of ligated reaction mix as per standard protocol. Screening of recombinant clones was done by colony PCR and RE digestion. The *PIMT* gene cloned in pET28c plasmid was confirmed by sequencing by using plasmid as template and T7 terminator and promoter as sequencing primers. Further, the recombinant clone was confirmed by SDS-PAGE after addition of isopropyl β-D-thiogalactopyranoside in the bacterial culture at the final concentration of 0.5 mM for 3 h at 25°C. The sequencing results were assembled and analyzed using DNASTAR package analysis software. The final sequence was aligned with the available *PIMT* gene of *S. enterica* serovar Typhimurium str. LT2. The sequence was submitted to NCBI Genbank. Further, the nucleotide sequence was subjected to nucleotide BLAST to compare for sequence similarities with other sequences available in the NCBI database (<http://blast.ncbi.nlm.nih.gov>).

Results

Salmonella Typhimurium strain of poultry origin was used in the current study. The strain showed typical morphological, cultural and serological characteristics of the *Salmonella*. On HEA plate, the isolate produced smooth, transparent, black centered colonies with greenish periphery (Figure-1). In slide agglutination test, the organism reacted with the standard positive serum.

The genomic DNA from *Salmonella* was isolated, and the ratio of OD_{260/280} was 1.97, which indicates the DNA was pure. The PCR amplified *PIMT* was purified and the concentration of the PCR product was ~20 ng/µl. This *PIMT* insert was directionally cloned into an expression vector pET28c and was PCR amplified using *PIMT*-pET28c as template. This DNA migrated as a single band of approximately 627bp in 1% agarose gel (Figure-2 and lane 1 and 2) as that of amplified insert DNA (Data not shown). The nucleotide sequence was confirmed by restriction digestion and sequencing and was submitted to NCBI GenBank (accession no. KJ575536). Sequencing data were

Table-1: Designed primers of *PIMT* of *S. enterica* subsp. *enterica* serovar Typhimurium.

Forward primer, PIMT F (<i>NheI</i>)	5'-ATATAT GCTAGC ATG GTA AGT GGA CGT GTA CAG-3'
Reverse primer, PIMT R (<i>BamHI</i>)	5'-ATATAT GGATCC GGC CAG CTC TCC CTT GAC-3'

PIMT: Protein *L*-isoaspartyl *O*-methyl transferase,
S. enterica=*Salmonella enterica*

analyzed using DNA star software and NCBI home page and 100% homology between *PIMT* sequences from *Salmonella* Typhimurium strain E-2375 (used in the current study) and retrieved *S. Typhimurium* LT2 *PIMT* (Figure-3). After SDS-PAGE following induction a band migrating ~28 kDa was observed in induced lane that was absent in the corresponding uninduced control lane (Figure-4).

The sequence when subjected to nucleotide BLAST search revealed a high degree of similarity with other *Salmonella* serovar (Table-2). The *PIMT* gene is highly conserved in most of the pathogenic serovars of *Salmonella* as evidenced from the sequence analysis. The obtained sequences shown 100% similarity with *PIMT* gene sequences of *Salmonella* Typhimurium



Figure-1: Growth of *Salmonella* Typhimurium E-2375 strain on Hektoen Enteric Agar plate. The cells were streaked and incubated at 37°C for overnight. Isolated colonies are depicted by arrows.

DT104 (HF937208), *Salmonella* Typhimurium str. 08-1736 (CP006602), *Salmonella* Typhimurium str. D23580 (FN424405) and *Salmonella* Typhimurium SL1344 (FQ312003) and 99% similarity with str. DT2 (HG326213), *S. Newport* str. SL254 (CP001113), *Salmonella* Bareilly str. CFSAN000189 (CP006053), *Salmonella* Enteritidis str. EC20100325 (CP007360), *Salmonella* Anatum str. ATCC BAA-1592 (CP007531), *Salmonella* Heidelberg str. SL476 (CP001120) and *S. Enteritidis* str. EC20100130 (CP007358). The expressed protein was isolated from

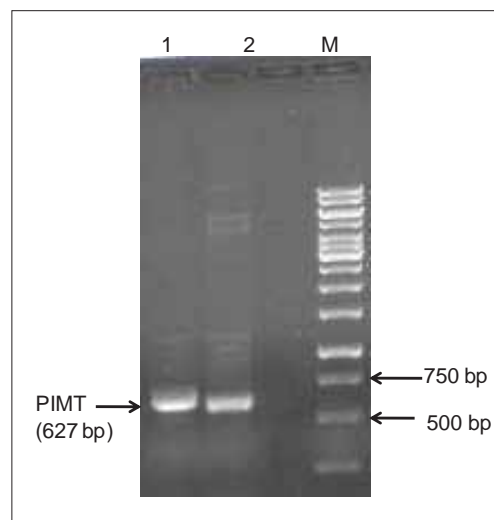


Figure-2: Polymerase chain reaction (PCR) amplification of *Salmonella* Typhimurium protein L-isoaspartyl O-methyl transferase using plasmid DNA template. The amplified products were analysed on 1% agarose gel. Lane M: 1 kb DNA ladder, Lane 1 and 2: PCR amplified products marked by arrow.

Table-2: Results of nucleotide blast showing similarity of *PIMT* gene of *S. enterica* serovar Typhimurium strain E-2375 with other *Salmonella* serovars.

Accession number	Description	Maximum score	Total score	Query coverage %	Maximum indent %
HF937208	<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium DT104 main chromosome, complete genome	1153	1153	100	100
CP006602	<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium str. 08-1736, complete genome	1153	1153	100	100
FN424405	<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium str. D23580 complete genome	1153	1153	100	100
FQ312003	<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium SL1344 complete genome	1153	1153	100	100
HG326213	<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium str. DT2, complete genome	1147	1147	100	99
CP001113	<i>S. enterica</i> subsp. <i>enterica</i> serovar Newport str. SL254, complete genome	1136	1136	100	99
CP006053	<i>S. enterica</i> subsp. <i>enterica</i> serovar Bareilly str. CFSAN000189, complete genome	1120	1120	100	99
CP007360	<i>S. enterica</i> subsp. <i>enterica</i> serovar Enteritidis str. EC20100325 genome	1109	1109	100	99
CP007531	<i>S. enterica</i> subsp. <i>enterica</i> serovar Anatum str. ATCC BAA-1592, complete genome	1131	1131	100	99
CP001120	<i>S. enterica</i> subsp. <i>enterica</i> serovar Heidelberg str. SL476, complete genome	1125	1125	100	99
CP007358	<i>S. enterica</i> subsp. <i>enterica</i> serovar Enteritidis str. EC20100130, complete genome	1109	1109	100	99

PIMT: Protein L-isoaspartyl O-methyl transferase, *S. enterica*=*Salmonella enterica*

Score	Expect	Identities	Gaps	Strand
1153 bits(624)	0.0	624/624(100%)	0/624(0%)	Plus/Plus
Query 122	ATGGTAAGTGGACGTGTACAGGCTCTTCTTGAACAATTGCGCGCGCAGGGGCATCAGAGAT	181		
Sbjct 1	ATGGTAAGTGGACGTGTACAGGCTCTTCTTGAACAATTGCGCGCGCAGGGGCATCAGAGAT	60		
Query 182	GAGCAGGTGCTTAATGCGCTTGTCTGCAGTGCCGCGCGAGAAATTTATTGATGAAGCGTTT	241		
Sbjct 61	GAGCAGGTGCTTAATGCGCTTGTCTGCAGTGCCGCGCGAGAAATTTATTGATGAAGCGTTT	120		
Query 242	GAACATAAGGCCTGGGAAAATATCGCTTTGCCGATAGGCCAGGGTCAGACGATTTTCGCAG	301		
Sbjct 121	GAACATAAGGCCTGGGAAAATATCGCTTTGCCGATAGGCCAGGGTCAGACGATTTTCGCAG	180		
Query 302	CCCTATATGGTGGCGCGAATGACGGAGCTGCTCGAACTGACGCCGCAATCCAGGGTGCTG	361		
Sbjct 181	CCCTATATGGTGGCGCGAATGACGGAGCTGCTCGAACTGACGCCGCAATCCAGGGTGCTG	240		
Query 362	GAAATTGGTACCGGTTCCGGCTATCAGACGGCGATTCTGGCGCATCTGGTACATCACGTT	421		
Sbjct 241	GAAATTGGTACCGGTTCCGGCTATCAGACGGCGATTCTGGCGCATCTGGTACATCACGTT	300		
Query 422	TGCTCCGTTGAGCGGATTAAGGGGCTGCAATGGCAGGCGCGTCGCCGCCTGAAGCAGCTC	481		
Sbjct 301	TGCTCCGTTGAGCGGATTAAGGGGCTGCAATGGCAGGCGCGTCGCCGCCTGAAGCAGCTC	360		
Query 482	GATTTACATAATGTTTCTACCCGTCATGGCGATGGCTGGCAAGGCTGGCAGGCGCGTGCG	541		
Sbjct 361	GATTTACATAATGTTTCTACCCGTCATGGCGATGGCTGGCAAGGCTGGCAGGCGCGTGCG	420		
Query 542	CCATTTGACGCTATCATTGTGACGGCCGCGCCGCCGAAATTCCTACCGCGCTCATGGCA	601		
Sbjct 421	CCATTTGACGCTATCATTGTGACGGCCGCGCCGCCGAAATTCCTACCGCGCTCATGGCA	480		
Query 602	CAGTTGGATGAAGGCGGCATTCTTGTCTGCCCGTGGGCGATGAGCAGCAGTTTTTGA	661		
Sbjct 481	CAGTTGGATGAAGGCGGCATTCTTGTCTGCCCGTGGGCGATGAGCAGCAGTTTTTGA	540		
Query 662	CGCGTGCCTCGCCGGGGCGGCGAATTTATTATCGATAACCGTGGAGGCCGTTTCGCTTCGTC	721		
Sbjct 541	CGCGTGCCTCGCCGGGGCGGCGAATTTATTATCGATAACCGTGGAGGCCGTTTCGCTTCGTC	600		
Query 722	CCGTTAGTCAAGGGAGAGCTGGCC	745		
Sbjct 601	CCGTTAGTCAAGGGAGAGCTGGCC	624		

Figure-3: Sequence analysis of protein *L*-isoaspartyl *O*-methyl transferase (*PIMT*) from positive clones resembling 100% homology: Query-*PIMT* sequence obtained by sequencing, subject-retrieved *PIMT* sequence of *S. Typhimurium* LT2 strain from NCBI.

the soluble fraction and migrated as around 28 kDa band on SDS gelatin histidine was fused with *PIMT*.

Discussion

The importance of *PIMT* has been demonstrated in *E. coli*. The *E. coli* Δ *pimt* (mutant *PIMT*) showed compromised survival in stationary phase of growth when subjected to oxidative stress [9]. This protein repairing enzyme is highly conserved across the phylogenetic domain including eubacteria, archaeobacteria, protozoa, fungi, nematodes, mammals and plants [16]. The structural sequence similarities reveal the same functional characterization of almost all *Salmonella* serovars. To know more about *PIMT* role and establishment of the importance of this enzyme, cloning and sequencing is primary attempt that can be further helpful to produce

the *PIMT* protein for its functional characterization by identifying its target to establish its virulence properties.

Conclusion

Salmonellosis caused by *Salmonella* Typhimurium is an important food borne intracellular pathogen and disease spreads mainly due to consumption of contaminated poultry products. Intra phagocytic survival of *Salmonella* Typhimurium depends upon its ability to quench phagocyte generated oxidants and repair of macromolecules. Although this bacterium harbors several primary antioxidant enzymes, its survival inside the macrophages at least in part depends upon repair of damaged proteins. The repair of the isoaspartyl proteins under oxidative stress is crucial to reactivate their

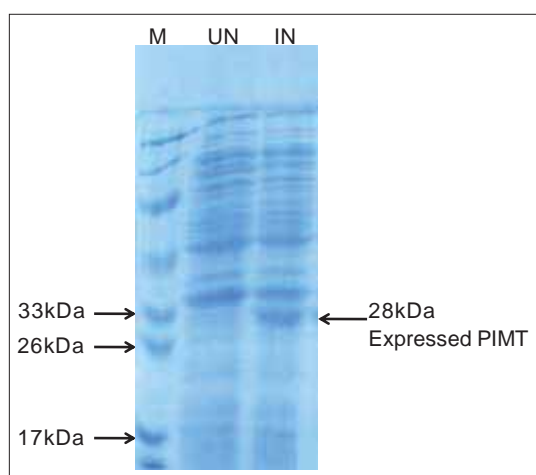


Figure-4: SDS-PAGE analysis of *Escherichia coli* bacterial cell lysate following induction of IPTG. Lane UN and IN are uninduced and induce cultures. Lane: M is the protein marker.

functions. This *PIMT* enzyme is responsible for the repair of the same to rescue its functions. This *PIMT* enzyme of *Salmonella* is highly conserved in the nucleotide sequence among the pathogenic *Salmonella* serovars. The similarity in the sequences of the cloned product with other *Salmonella* serovars suggests its importance in the utility of *PIMT* enzyme for its restoration of protein function as a mean of intraphagosomal survival. It is not yet cleared whether *PIMT* can be used as detective antisera for confirmation of clinical cases of *Salmonella*, which needs further experimental study.

Authors' Contributions

SKD, TKG and MM designed the experiments. SKD and DPH carried out the experimental work. MM, TKG and SKD were involved in scientific discussion and analysis of the data. SKD and MK drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

References

- Christou, L. (2011) The global burden of bacterial and viral zoonotic infections. *Clin. Microbiol. Infect.*, 17(3): 326-330.
- Kingsley, R.A., Msefula, C.L., Thomson, N.R., Kariuki, S. and Holt, K.E. (2009) Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res.* 19(12): 2279-2287.
- Velge, P., Cloeckert, A. and Barrow, P. (2005). Emergence of *Salmonella* epidemics: The problems related to *Salmonella enterica* serotype enteritidis and multiple antibiotic resistance in other major serotypes. *Vet. Res.*, 36(3): 267-288.
- Wales, A.D. and Davies, R.H. (2011) A critical review of *Salmonella* Typhimurium infection in laying hens. *Avian. Pathol.*, 40(5): 429-436.
- Parry, C.M. and Threlfall, E.J. (2008) Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. *Curr. Opin. Infect. Dis.*, 21(5): 531-538.
- Ibarra, J.A. and Steele-Mortimer, O. (2009) *Salmonella*- the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cell. Microbiol.*, 11(11): 1579-1586.
- Haraga, A., Ohlson, M.B. and Miller, S.I., (2008) *Salmonella* interplay with host cells. *Nat. Rev. Microbiol.*, 6(1): 53-66.
- Mahawar, M., Tran, V., Sharp, J.S. and Maier, R.J. (2011) Synergistic roles of *Helicobacter pylori* methionine sulf-oxide reductase and GroEL in repairing oxidant-damaged catalase. *J. Biol. Chem.*, 286(21): 19159-19169.
- Li, C. and Clarke, S. (1992) Protein methyltransferase specific for altered aspartyl residues is important in *Escherichia coli* stationary-phase survival and heat-shock resistance. *Proc. Natl. Acad. Sci.*, 89(20): 9885-9889.
- Lowenson, J.D. and Clarke, S., (1992) Recognition of D-aspartyl residues in polypeptides by the erythrocyte L-isoaspartyl/D-aspartyl protein methyltransferase. Implication for the repair hypothesis. *J. Biol. Chem.*, 267(9): 5985-5995.
- Chatterjee, T., Pal, A., Chakravarty, D., Dey, S., Saha, R.P. and Chakrabarti, P. (2013) Protein L-isoaspartyl-O-methyltransferase of *Vibrio cholerae*: Interaction with cofactors and effect of osmolytes on unfolding. *Biochimie.*, 95(4): 912-921.
- Verma, P., Singh, A., Kaur, H. and Majee, M. (2010) Protein L-isoaspartyl methyltransferase 1 (*CaPIMT1*) from chickpea mitigates oxidative stress-induced growth inhibition of *E. coli*. *Planta.*, 231(2): 329-336.
- Fang, P., Li, X., Wang, J., Xing, L., Gao, Y., Niu, L. and Teng, M. (2010) Crystal structure of the protein L-isoaspartyl methyltransferase from *Escherichia coli*. *Cell. Biochem. Biophys.*, 58: 163-167.
- Agarwal, R.K., Bhilegaonkar, K.N., Singh, D.K., Ashok, K. and Rathore, R.S. (2003) Laboratory manual for the isolation and identification of foodborne pathogens. Indian Veterinary Research Institute, Izatnagar, Bareilly, India. p100.
- Sambrook, J. and Russel, D.W. (2001) In: Molecular Cloning, a Laboratory Manual. 3rd ed. Vol. I. CSHL Press, USA. p8.2-8.7.
- O'Connor, C.M. (2006) Protein L-isoaspartyl/D-aspartyl O-methyltransferases: Catalysts for protein repair. In protein methyltransferases. *Enzymes.*, 24: 383-431.
