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Structure prediction of caffeine demethylating enzyme from *Pseudomonas alcaligenes*

V.R.Sarath Babu[1], M.S. Thakur[3] and Sanjukta Patra[2+]

[1]Fermentech Biologics, Bhimavaram, India-534202
[3]Fermentation Technology and Bioengineering Department, CFTRI, Mysore, India
[2]Dept. of Biotechnology, IIT, Guahati, India-781039

Abstract. Along with its stimulating effects caffeine has various deleterious effect which demands for the process of decaffeination. The present work aims at the structure prediction of decaffeinating enzyme from Pseudomonas. The purified enzyme was subjected to LCMS. and analyzed using MASCOT peptide mass analysis, the peptide sequences thus obtained were used for deriving the amino acid sequence of the protein. The amino acid sequence of the 1N-demethylase was found to contain 3 D-P bonds at Asp143-Pro144, Asp183-Pro184 and Pro214-Asp215. The secondary structure of caffeine demethylase was predicted and results showed 13.93 % of the protein is beta pleated, 27.55% alpha helical and 58.51 % of the protein occurs as random coils. The tertiary structure was predicted using **1ndo** (pdbid) as template. The protein is organized into a domain containing a six membered β-pleated sheet barrel which is present in the core of the enzyme, concealed in the hydrophobic pockets. A 3 membered β -sheet saddle is also present in the enzyme. Two histidines and one isoleucine residue appears to be involved in substrate binding.

Keywords: Caffeine, decaffeination, caffeine demethylase, 1ndo, tertiary structure

1. Introduction

Caffeine has deleterious effects on cardiac patients and women (James, 1997; Waring et. al., 2003). Reports are also available on the effects of caffeine on health and of its toxic effects to animals and plants (Pincheira et.al., 2003; Meyer et.al., 2004). Decaffeination is being carried out widely in beverages because of the growing belief that the chronic ingestion of caffeine can have adverse effects on health. However conventional methods of decaffeination have various disadvantages demanding for biodecaffeination. However, till date the structure of caffeine demethylating enzyme has not been predicted. The present work intends to predict the structure of caffeine demethylating enzyme from Pseudomonas alcaligenes.

2. Materials and Methods

2.1. Materials

Caffeine (99.9%), was procured from M/s Sigma-Aldrich, St. Louis, USA. All reagents were of the highest purity and were procured from standard sources.

2.2. Methods

3. Induction and purification of enzyme

Bacterial culture isolated from soil samples of tea and coffee gardens was identified as *Pseudomonas alcaligenes*. It was grown in the optimized growth medium containing sucrose (30 $g.L^{-1}$), caffeine (1.5 $g.L^{-1}$),

+ Corresponding author. Tel.: + (91-361-2582213); fax: +(91-361-2582249).
E-mail address: sanjukta@iitg.ernet.in

yeast extract (15 g.L^{-1}), peptone (30 g.L^{-1}) and ammonium sulphate (15 g.L^{-}1)., at 30^{o}C, and pH 7.0, inoculum of 5 %w/v and 150rpm. Biomass accumulated after 24 hours were harvested at 16,000g for 20 min at 0-4^{0}C. The pellet was aseptically transferred into a 500ml flask containing 100ml of caffeine liquid media (CLM) containing 1g.L^{-1} caffeine and incubated at 30^{o}C on shaker for a period of 48hrs for inducing the cells to degrade caffeine. Induced cells were harvested by centrifugation at 12000 g for 30 minutes at 4^{o}C, washed with ice cold buffer (Tris-Cl, 50 mM; pH 6.8) and frozen at –20^{o}C. 10 grams of the frozen pellet was thawed into 100 ml of Lysis buffers at 37^{o}C for 1 hour. The lysate was centrifuged at 12000 g for 30 minutes to separate cell debris. The supernatant obtained designated as crude enzyme was used for further purification by ammonium sulphate precipitation Sephadex G -100 column. Further purification was obtained by a phenyl sepharose column and purity of enzyme was checked by SDS PAGE.

3.1. Liquid chromatography with mass spectroscopic (LC-MS) analysis of caffeine demethylase enzyme:

Active fractions of purified enzyme were pooled, concentrated and subjected to trypsin digestion (Stone and Williams, 1996) and used for LC MS analysis, injected into a Q-Tof Ultima mass spectrometer equipped with a liquid chromatograph connected to a C8 column (Shevchenko et.al., 1996). The chromatographic separation of the protein was done with an increasing gradient of 70% acetonitrile containing 0.1% TFA and water containing 0.1% TFA (Papayannopoulos, 1995). The peaks were directly injected into the mass spectrophotometer attached with electro spray ionization injector in the positive mode. The collision energy was 10.0 kV. The mass peaks obtained were analyzed by mass lynx software and the masses of the proteins were calculated using the mass finder option. The peptides obtained by tryptic digestion were also analysed in the ESI positive mode and the sequencing of the mass fragments was done by using MASCOT software (Matrix Science Inc. Boston, USA; www.matrixsciences.com).

4. Results and Discussion

4.1. Induction and purification of enzyme

The microbial culture isolated from soil samples of tea and coffee plantation was identified as *Pseudomonas alcaligenes*. The enzyme was salted out at an ammonium sulphate concentration of 30-60 % w/v. Gel permeation chromatography on Sephadex G-100 column led to further purification, the enzyme eluted from the gel in the fractions showed a 2 fold increase in the purity. The enzyme was found to bind to phenyl sepharose matrix and was eluted with 0.5 M ammonium sulphate in the buffer.

4.2. Characterization of caffeine demethylase by LC-MS analysis

The peptide mass finger prints were analyzed using MASCOT peptide mass analysis software developed by Matrix Life Sciences Ltd (www.matrixsciences.com). The peptide sequences thus obtained were used for deriving the amino acid sequence of the protein using bioinformatics and method developed by Shevchenko, et. al., (1996). The probable genomic DNA for the protein was available from a Japanese patent (Imai et.al., 1996). The gDNA sequence is as follows: >gi|2175607|dbj|e07469.1|

The coding region of this protein was deduced by using the 6 frame SOPMA analysis (www.ncbi.nlm.nih.gov/SOPMA).

The deduced Sequence of the protein is:

MEQTINNNDRKYLRHFWHPVCTVTELEKAHPSSLVPIGVKLLNEQLVVAKLSGQYVAMHDRCAHR
SAKLSLGTIANDRLQCPYHGWQYDTEGACKLVPACPNSPIPNRAKVQRFDCEERYGLIWVRLDSSY
ACTEIPYFSAASDPKLRVVIQEPYWWNATAERRWENFTDFSHFAFIHPGTLFDPNNAEPPIVPMDRF
NGQFRFVYDTPEDMAVPDQAPIGSFSYTCSMPFAINLEVAKYSSNSLHVLFNVSCPVDDSTTKNFLL
FAREQADDSDYLHIAFNDLVFAEDKPVIESQWPKMLRLMKFRLSRIKSRSSIENGCGN

Using peptide cutter software, the probable cleavage sites and resultant fragments were obtained. The peptide sequences derived from LC MS analysis of the protein were compared by sequence alignment tools (Clustal w). The sequence of caffeine demethylase deduced by us has more similarity to the one reported in the Japanese patent, which is concluded as a caffeine 1N-demethylase producing theobromine.

5. Structure prediction of the enzyme

5.1. Secondary structure

The secondary structure of caffeine demethylase was predicted using Hierarchical Neural Network Software (Bairoch, et. al., 1997).

10 20 30 40 50 60 70

Fig. 1: Hierarchical neural network result for caffeine demethylase for prediction of secondary structure.

MEQTINNNDRKYLRHFWHPVCTVTELEKAHPSSLGPIGVKLLNEQLVVAKLSGQYVAMHDRCAHR
SAKLSCccccccchhhhhhhhccccceeeecccccccccchhhhhhhhhhhhhhhhcccchhhhhhhhhhchcccc
LGTIANDRLQCPYHGWQYDTEGACKLVPACPNSPIPNRAKVQRFDCEERYGLIWVRLDSSYACTEI
PYFSCchecccccccccccccccccccceecccccccccccchhhhcchccccceeeeeecccccccccceeAASDPKLRVVIQEPY
WWNATAERRWENFTDFSHFAFIHPGTLFDPNNAEPPIVPMDRFNGQFRFVYDTPECccccceeeeeecccc
ccchhhhhhhhhcccceeeeecccccccccccccccccccccccceeeeecccDMAVPDQAPIGSFSYTCSMPFAINLEVAK
YSSNSLHVLFNVSCPVDDSTTKNFLLFAREQADDSDYLHIACccccccccccccccccccchchhhhhhhcccceee
eeeecccccccchhhhhhhhhcccccchheeeFNDLVFAEDKPVIESQWPKMLRLMKFRLSRIKSRSSIENGCGNH
chheeccccccchhhhhhhhhhhhhhhhhecccccccccccc

C= Random coil
H= Helix
E= Beta pleated Sheet

Results show that 13.93 % of the protein is beta pleated in nature, 27.55% alpha helical and 58.51 % of the protein occurs as random coils (Fig. 2 & Fig. 3).

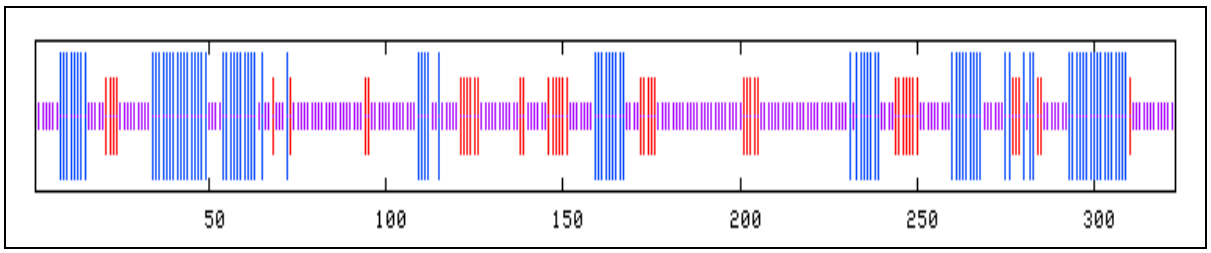

Fig. 2: Graphical representation of the predicted secondary structure of caffeine demethylase.

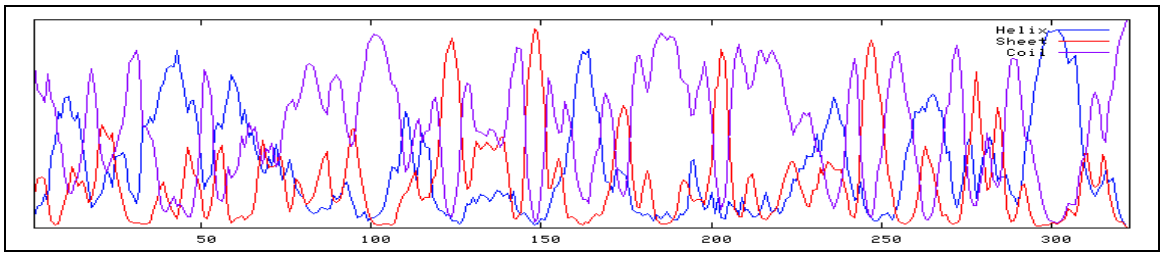

Fig. 3: Graphical representation of secondary structure of caffeine demethylase (HNN).

Figure 4. represents Ramachandran plot of caffeine 1N-demethylase. From the plot it can be observed that 79.7% of the residues lie in the core region of the protein and 16.1% lie in the allowed region. Number of glycines is 12 which accounts for 3.7% of the protein and 23 prolines are present in the sequence accounting for 7.2% of the enzyme.

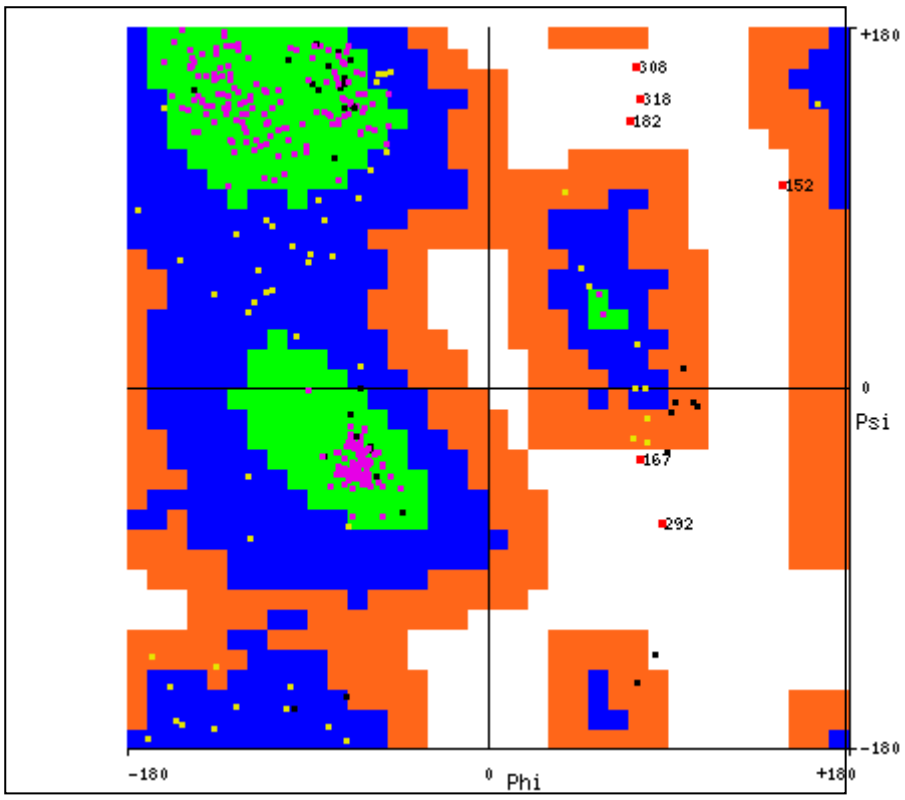

Fig. 4: Ramachandran plot of caffeine demethylase enzyme.

CORE ALLOWED GENEROUS DISALLOWED PRO GLY

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.

residues in CORE:	79.7 % (228)
residues in ALLOWED:	16.1 % (46)
residues in CORE+ALLOWED:	95.8 %
residues in GENEROUS:	2.1 % (6)
residues in DISALLOWED:	2.1 % (6)
number of GLY:	12 (3.7 %)
number of PRO:	23 (7.2 %)

DISALLOWED region	GENEROUS region	ALLOWED region	CORE region

5.2. Tertiary structure prediction

The tertiary structure of caffeine 1N-demethylase was predicted using fold recognition server and the template used was **1ndo** (pdbid). Figure 5 represents the predicted structure of caffeine demethylase. Based on the predicted structure, the protein is organized into a domain containing a six membered β-pleated sheet barrel. β-sheet barrels in enzymes are usually involved in the channeling of the substrate to the active site and in the solvent accessibility. These are present in the core of the enzyme, concealed in the hydrophobic pockets of the enzyme. A 3 membered β -sheet saddle is also present in the enzyme. Two histidines and one isoleucine residue appear to be involved in the binding of caffeine to the enzyme. The function of 2 extended sheets towards the surface of the protein is not known. The α-helices reside on the surface of the protein indicating they are composed mostly of hydrophilic residues. It is a common feature of the NADPH binding sites and Fe-S centers are present in the periphery and mostly comprise of α-helices as in the case of other demethylases like vanillate demethylase, lanosterol demethylase etc., which also have rieke Fe-S centers, involved in NADPH/NADH oxidation (Bernhardt, 1975; Buswell and Ribbons, 1988; Cartwright and Smith,

1967). NADPH binding in these proteins occurs at the surface of the protein in α- helices of the surface domains of the protein. The Fe-S center (Figure 5.) with a prophyrin ring is involved in the binding of oxygen to the enzyme. The rest of the protein is organized as random coils. The random coils are mostly involved in the stabilization of the back bone of the protein. They are mostly comprised of proline residues, which are responsible for bend in the coils.

Fig. 5: Predicted 3D structure of caffeine demethylase enzyme.

6. Conclusions

The enzyme involved in 1N-demethylation of caffeine was characterized by LC MS analysis and bioinformatics tools. The tertiary structure of the enzyme has been predicted using bioinformatics approach.

7. References

[1] Bairoch, A., Bucher, Hofmann, K., (1997), The PROSITE database, its status in 1997. *Nucl. Acids Res*., **25:** 217-221.

[2] Bernhardt, F. H. P. H. S. H., (1975), *Eur. J. Biochem*. **57:**241–256.

[3] Buswell, J. A., Ribbons, D. W., (1988), *Methods. Enzymol.* **161:**294–301.

[4] Cartwright, N. J., Smith, A.R.W. *Biochem. J*. (1967) **102:**826–841.

[5] Imai, Y., Nakane, S., Koide, Y., United States Patent 5550041. (1996)

[6] James, J.E., *Lancet*. (1997) **349:**279–281.

[7] Meyer, L., Caston, J., Lieberman, H.R., *Behav. Brain. Res.* (2004) **149:**87–93.

[8] Papayannopoulos, I. A., Mass Spectromet. Rev. (1995) **14:** 49-73.

[9] Pincheira, J., L´opez-S´aez, J.F., Carrera, P., Navarreteb, M.H., Torre, C.D.L., *Cell. Biol. Int.* (2003) *27:837*–843.

[10] Rojas, J.B.U., Verreth, J.A.J., Amato, S., Huisman, E.S., *Bioresour. Technol.* (2003) **89:**267–274.

[11] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., *Anal. Chem*. (1996) **68:** 850-858.

[12] Stone, K.L., Williams, K.L., *The Protein Protocols Handbook*, eds. J.M. Walker, (1996) Humana Press Inc., Totowa, NJ, pp.415 ff.

[13] Waring, W.S., Goudsmit, J., Marwick, J., Webb, D.J., Maxwell, S.R.J., *Am. J. Hypertens.* (2003) 16:919–924.