Current Research Journal of Biological Sciences 3(1): 35-41, 2011
ISSN: 2041-0778

Submitted: October 22, 2010 Accepted: November 18, 2010 Published: January 15, 2011

Physiochemical, Functional and Structural Characterization of Wheat Germin Using *In silico* Methods

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Abstract: Wheat germin, an oxalate oxidase possesses high proteolytic and thermal stability but is poorly characterized. Three dimensional structure of wheat germin is also not available at PDB. The present paper uses bioinformatics approach to describe the physiochemical, functional and structural properties of wheat germin. The three dimensional structure of wheat germin is predicted by homology modelling using the highly homologous barley germin as template. The model was evaluated with PROCHECK, VERIFY 3D, WHAT IF, ERRAT and ProSA programs. Model visualization and analysis was done with Swiss-PDB viewer. Nature of active site and key features of the model responsible for stability of enzyme are also discussed.

Key words: Homology modelling, oxalate oxidase, properties, stability, structure

INTRODUCTION

It is now well established that germin, a marker of embryonic development in wheat is an oxalate oxidase (OxOx), present at high levels in the extracellular matrix of grasses (Lane *et al*., 1993; Lane, 2000). Although germins, germin-like proteins (ubiquitous proteins that lack OxOx activity) and seed storage proteins, all three belong to cupin superfamily characterized by conserved beta- barrel core structure and a signal peptide but structurally germins are more similar to germin like proteins (Khuri *et al*., 2001; Patnaik and Khurana, 2001).

Oxalate oxidase activity of germins catalyzes the oxidative breakdown of oxalate to carbon dioxide and hydrogen peroxide:

$$(COOH)_2 + O_2 \rightarrow 2CO_2 + H_2O_2$$

Released H_2O_2 acts as a signal for plant defense and signaling, making the plant resistant to fungal infections (Lane, 2002).

So far, barley OxOx is the best characterized germin used commercially for clinical determination of oxalate in biological samples (Chandran *et al*., 2001; Petrarulo *et al*., 1994). Wheat germin/OxOx also has huge potential for commercial applications as it shows unusual resistance to proteases, possess high heat stability and resists dissociation by detergents (Carter and Thornburg, 1999). Expression of wheat germin in transgenic tobacco (Berna and Bernier, 1997), *E. coli* (Cassland *et al*., 2004) and *Pichiapastoris* (Pan *et al*., 2007) has already demonstrated the suitability of large scale production of OxOx for biomedical applications. However till date little efforts have been made to characterize this unique enzyme. Recently report on characterization of OxOx from wheat seedling suggested it to have a pentameric structure with a mass of 179 kDa (Yihong and Zhenfei, 2009).

Three dimensional structure of a protein is an invaluable aid to understand the details of a particular protein. Earlier, a three dimensional model of wheat germin based on the structure of distantly related (>30% identity) seed storage protein vicilin predicted the presence of three His residues at the active site (Gane *et al*., 1998). Homology modelling based on the crystal structure of any close homolog such as barley germin, will build a three dimensional model for wheat germin with increased accuracy, reliability and statistical robustness.

Hence, the present study predicts some of the properties of wheat germin and proposes a model of its three dimensional structure. The study will be valuable to understand the structural features and molecular function of wheat germin and will raise the prospects of its potential commercial or academic use.

MATERIALS AND METHODS

The study was conducted by the author in the Dept. of Botany starting from June, 2010 using Intel Pentium 4, 2.40 GHz, 32 bit operating system.

Retrieval of target and template sequence: The amino acid sequence of wheat germin was obtained in fasta format from the Protein sequence database of NCBI (GenBank Id: AAA34271.1) (Lane *et al*., 1991). Similarity between the retrieved sequence and other sequences was found with Blastp 2.2.24 search (Altschul *et al*., 1990) within the Protein Data Bank (PDB) proteins. Parameter values for BLAST were set as default.

Physiochemical and functional characterization: Molecular weight, theoretical pI, total number of negatively (Asp+Glu) and positively (Arg+Lys) charged residues, extinction coefficients (Gill and Von Hippel, 1989), instability index (Guruprasad *et al*., 1990), aliphatic index (Ikai, 1980) and grand average of hydropathicity (GRAVY) (Kyte and Doolittle, 1982) was computed using Expasy'sProtParam Proteomics server (Gasteiger *et al*., 2005). For functional characterization disulfide bonds were predicted by the Cys_REC tool from softberry, secondary structures were calculated with SOPMA (Self Optimized Prediction Method with Alignment) (Geourjon and Deléage, 1995). DSSP (Kabsch and Sander, 1983) was also used to calculate secondary structure elements from the three dimensional coordinate.

Homology modeling: Crystal structure of barley germin (PDB Id: 1FI2) was selected as template to create the three dimensional model for wheat germin (Woo *et al*., 1998; Woo *et al*., 2000). The homology modelling program, Modeller 9v8 (Sali and Blundell, 1993) was used to generate a total of 10 models of target protein. The models were viewed with Swiss PDB viewer (Guex and Peitsch, 1997).

Model refinement and evaluation: The models constructed were solvated and subjected to energy minimization using the steepest descent and conjugate gradient technique to eliminate bad contacts between protein atoms and structural water molecules. Computations were carried out *in vacuo* with the GROMOS 96 43B1 parameters set, implementation of Swiss-PDB Viewer. The stereochemical quality and accuracy of the predicted models was evaluated with PROCHECK (Laskowski *et al*., 1996) by Ramachandran plot analysis (Ramachandran *et al*., 1963). The best model was selected on the basis of overall G-factor, number of residues in core, allowed, generously allowed and disallowed regions. The selected model was further put to analysis by VERIFY 3D (Eisenberg *et al*., 1997), WHAT IF (Vriend, 1990) and ERRAT (Colovos and Yeates, 1993) programs. ProSA (Wiederstein and Sippl, 2007) was used for the display of Z-scores and energy plots for both wheat and barley germin. In the last step, the homology model was visualized and analyzed with Swiss-PDB viewer (Guex and Peitsch, 1997).

Analysis of the predicted model: The modelled three dimensional structure of wheat germin was analyzed using Deep View Swiss PDB viewer (Guex and Peitsch, 1997). Quality of the homology model was judged by selecting residues which make clashes in their existing position and by coloring protein problems, B-factor and threading energy. Active site residues and key features of the model responsible for its stability were also analyzed.

Table 1: Comparison of properties of wheat and barley germin as predicted by ProtParam program

		Proteins	
S. No.	Parameters	Wheat germin	Barley germin
1.	Sequence length	201a.a.	201a.a.
2.	Molecular weigth (nonglycosylated forms)	21204.3	21203.2
3.	Theoretical pI	7.80	5.52
4.	-R*	16	20
5.	+R*	17	16
6.	Extinction coefficients** (M^{-1} cm^{-1} at 260 nm)	7115-6990	8605-8480
7.	Instability index	24.13	21.86
8.	Aliphatic index	85.77	83.33
9.	GRAVY	0.049	-0.003

*: -R: Total number of negative residues; +R: Total number of positive residues

**: First value is based on the assumption both cysteine from cystines and the second assumes that both cysteine residues are reduced

RESULTS AND DISCUSSION

The 224 amino acid long sequence of wheat germin retrieved from Protein: sequence database of NCBI (GenBank Id: AAA34271.1) (Lane *et al*., 1991) had a putative signal peptide of 23 amino acids at N-terminus that targets the protein to the cell wall and a mature peptide with 201 amino acid residues. The sequence of mature protein was blasted against the PDB database for proteins with similar sequence and known three dimensional structure using compositionally adjusted substitution matrices. Two proteins, one a recombinant OxOx from barley expressed in *Pichia pastoris* (PDB Id: 2ETE) (Opaleye *et al*., 2006) and the other, a sequence of barley germin (PDB Id: 1FI2) (Woo *et al*., 1998) shared 92% residue identity with the target sequence. Barley germin was finally selected as template to model the structure of wheat germin as it had a better resolution of 1.60 Aº compared to that of 1.75 Aº for recombinant OxOx.

Physiochemical characterization: Physiochemical properties of target and template protein computed using ProtParam tool are given in Table 1. Template protein was subjected to ProtParam analysis for the purpose of its comparison with target protein. The computed isoelectric point (pI value) for wheat germin was 7.80, hence can be considered as basic in character whereas a pI value of 5.53 for barley germin indicates its acidic character. The computed isoelectric point will be useful for separating the protein on a polyacrylamide gel by isoelectric focusing. The extinction coefficient of a protein as calculated by the program depends on the molar extinction coefficient of Tyr, Trp and Cys residues. Difference in the extinction coefficient values for two proteins as evident from Table 1 was due to the difference in concentration of Tyr residues, the barley protein having two in contrast to that of a single Tyr residue in wheat germin. The extinction coefficient can be used to calculate

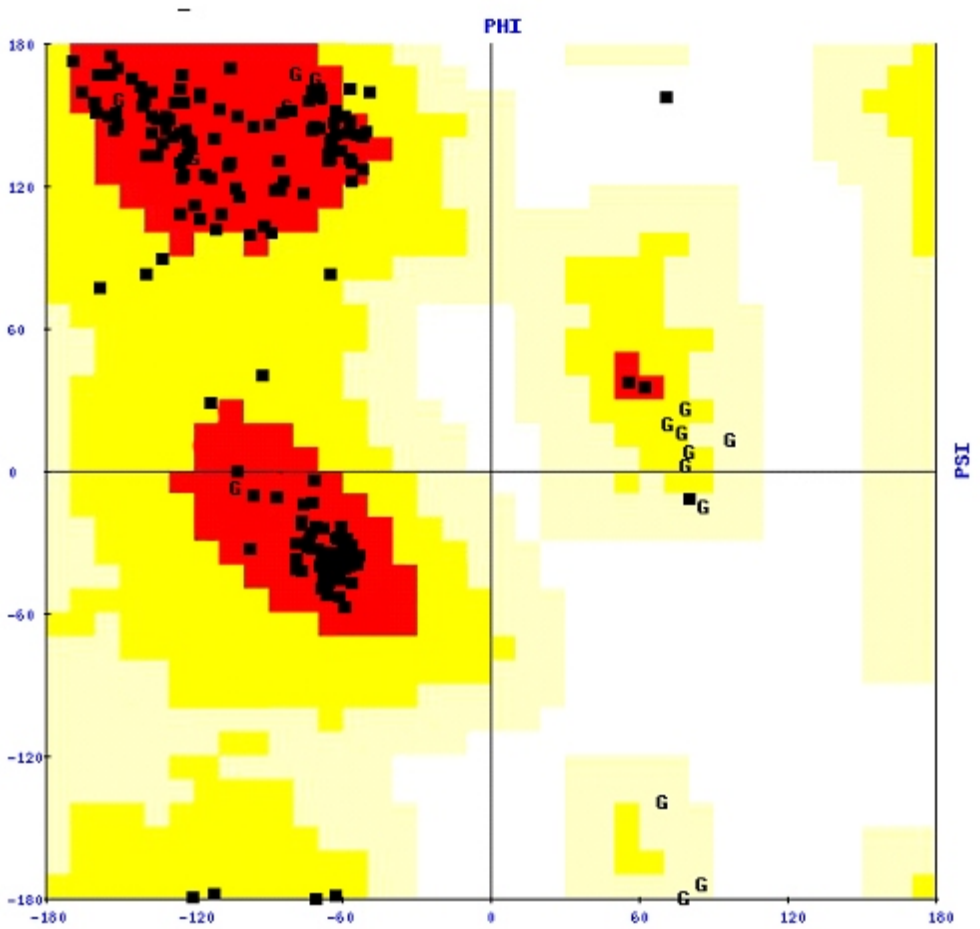

Fig. 1: Ramachndran plot analysis for wheat germin. The plot statistics are: Total number of residues-201 with 92% residues in the core region (red); 6.8 % residues in allowed (yellow); 0.6% in generously allowed (light yellow) and 0.6% residues (Ser 159) in the disallowed region (white). Number of glycine residues (labeled as G) is 21 and Number of Pro residues is 16

the concentration of a protein in solution. Instability index relies upon the occurrence of certain dipeptides along the length of the protein to distinguish between the unstable and stable protein. The value for instability index for both wheat and barley germin is less than 40, hence both can be predicted as stable proteins (Guruprasad *et al*., 1990). The aliphatic index refers to the relative volume of a protein that is occupied by aliphatic side chains and contributes to the increased thermostability of protein. Higher aliphatic index of wheat germin indicates that its structure is more stable than barley protein over a wide temperature range. Grand average of hydropathicity (GRAVY) index indicates the solubility of proteins: a positive GRAVY value for wheat germin designates it to be hydrophobic in nature whereas a negative GRAVY value for barley germin indicates more surface accessibility of the protein to interact with water.

Functional characterization: Disulfide bridges play an important role in the folding and stability of proteins. CYS_REC version 2 revealed the presence of one disulphide linkage at residue number 26 in both wheat and barley proteins. The secondary structure features as predicted by SOPMA showed that random coils dominated among secondary structure elements followed by extended strand, α helix and beta turns for both the proteins, the respective values being 45.27, 27.86, 18.91 and 7.9 for wheat germin and 43.78, 29.85, 16.42 and 9.95 for barley germin. The secondary structure was predicted by using default parameters (window width 17, similarity threshold:8 and number of conformational states: 4). The position of secondary - structure elements as searched by DSSP program was found to be essentially in agreement with that predicted by SOPMA.

Model building, refinement and evaluation: PROCHECK analysis: Three dimensional structure for wheat germin is not available hence, crystal structure of germin from barley was used as template to generate three dimensional coordinates for wheat germin. Ten models

generated by Modeller 9v7 were viewed with Swiss PDB viewer and energy minimized. Ramachandran plot analysis of the ten models was obtained by PROCHECK server. The best model in terms of stereochemical quality showed a positive overall G-factor value of 0.06 which indicates that geometry of the model corresponds to high probability confirmation with 99.4% residues in the allowed region of Ramachandran plot (Fig. 1). Only one residue i.e., Ser159 was found in the disallowed region of Ramachandran plot. It is generally accepted that if 90% residues are in the allowed region, the quality of the model is evaluated as good and reliable.

Verify 3D, what IF and ERRAT analysis: Verify 3D assigned a 3D-1D score of >0.2, predicting that the model is compatible with its sequence. Further validation by WHAT IF server assigned a Ramachandran Z-score of 0.29 and packing quality Z-score for all contacts to be -0.51. A positive Ramachandran Z-score and negative (between 0.0 to -2.0) packing quality Z-score was within expected range for well-defined structures. RMS Z-scores for bond angles and bond lengths as determined by WHAT IF was 1.030 and 0.608, respectively, which is very close to 1.0 suggesting high model quality. The amino acid environment was evaluated using ERRAT

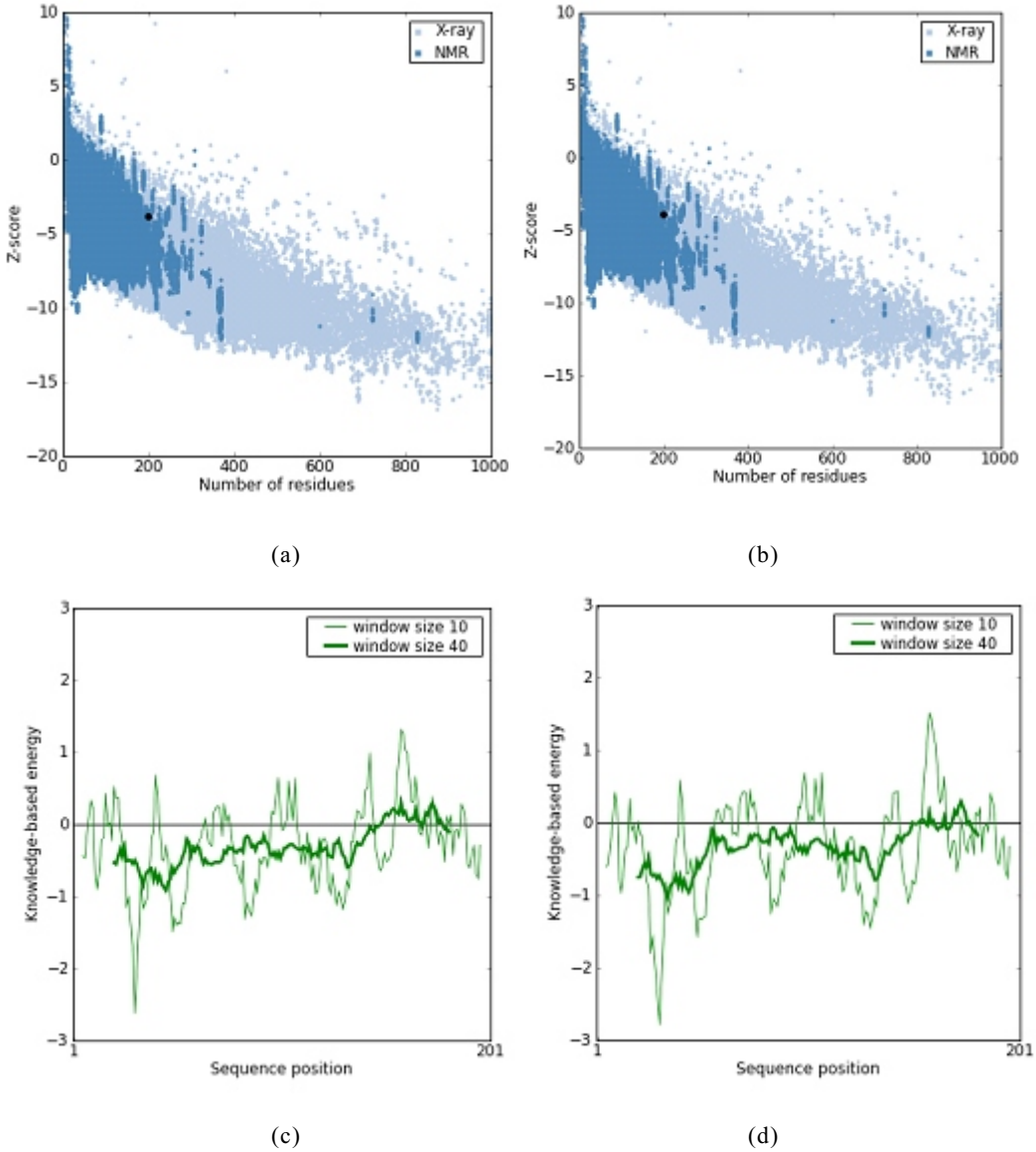

Fig. 2: ProSA-web service analysis of wheat and barley germin. (a and b) ProSA-web z-scores of all protein chains in PDB determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) with respect to their length. The z-scores of wheat (a) and barley (b) germin are highlighted as large dots. (c) Energy plot of wheat germin. (d) Energy plot of barley germin

plots, which assess the distribution of different types of atoms with respect to one another in the protein model and is used for making decision about its reliability. ERRAT showed an overall quality factor of 93.194, a result expected for crystallographic models with resolution >2.5 Aº.

ProSA analysis: ProSA was used to check three dimensional model of wheat germin for potential errors. The program displays two characteristics of the input structure: its z-score and a plot of its residue energies. The z-score indicates overall model quality and measures the deviation of the total energy of the structure with respect to an energy distribution derived from random conformations. As shown in Fig. 2 the Z-score for wheat germin is -3.82 which is very close to that calculated for barley germin (-3.9) and also well within the range of scores typically found for proteins of similar size indicating a highly reliable structure.. The energy plot shows the local model quality by plotting energies as a function of amino acid sequence position. In general, positive values correspond to problematic or erroneous parts of a model. Figure 2 displays a comparable energy plot for both the target and template structures.

Swiss-PDB viewer analysis of predicted model: Structural analysis: As judged by Deep View-Swiss PDB viewer none of the residues were found to make clashes in their existing position which means that the residues occupied locations that were not impossible due to steric hindrances. The threading profile of wheat germin was also very similar to that of template indicating good quality homology model. When protein problems were colored, Ser 159 was revealed to have bad backbone confirmation. Coloring backbone by "B-factor" showed that this region of target is not identical to the template and hence Ser 159 also lied in a stretch of Rama outliners. The predicted three dimensional structure of the final model shows an easily identifiable β-barrel fold having six beta strands arranged in an antiparallel fashion clearly forming the ligand binding site followed by three α-helices at C-terminus (Fig. 3). As the active site of enzyme homologs tend to be highly conserved, high homology between barley and wheat germin was exploited to confirm the nature of active site residues on the modelled structure. Barley germin is reported to have three three His (H^{88}, H^{90} and H^{137}) and one Glu (E^{95}) residue at the ligand binding site (Evdokimov *et al*., 2001). An analysis of the modelled structure revealed essentially the same residues at the β-barrel fold clearly forming a cluster ready to accept the ligand (Fig. 3). This is possible as both the proteins besides being highly homologous also bind to the same metal ion species i.e. manganese (Pan *et al*., 2007; Requena and Bornemann, 1999). Moreover, there was no other residue in the vicinity of the cluster within the

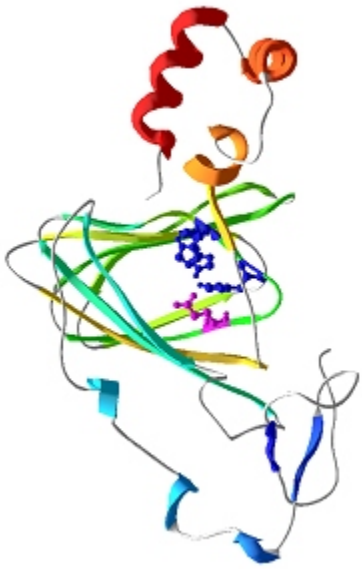

Fig. 3: Predicted three dimensional structure of wheat germin. The secondary structure elements are presented as ribbons. Residues nearest to N-terminus are blue and those nearest to C-terminus are red. Residues in between are assigned other colors spanning the visible spectrum. Ball and stick model of three His residues in blue (H^{88}, H^{90} and H^{137}) and one Glu residue in pink (E^{95}) is shown at the metal binding site

cavity of β-barrel fold. Earlier an analysis of the active site of wheat germin by Gane *et al.* (1998) did not reveal the presence of Glu residue at the active site. Superimposition of the model with the template (used as reference layer) showed a very low RMSD of 0.26 Aº, suggesting high similarity between them.

Stability analysis: Wheat germin possess high proteolytic and thermal stability. In fact, the proteolytic stability correlates with the thermal stability as most of the proteins are resistant to proteases in their native confirmation but are rapidly degraded after unfolding. The presence of tightly packed hydrophobic residues and in particular the formation of Ile clusters is regarded to play an important role in increasing the thermal stability of the protein (Evdokimov *et al*., 2001; Britton *et al*., 1995). In wheat germin 83.33% (5 out of total 6) of the Ile residues were clustered at β-barrel core (Fig. 4) which might play a role in making the protein thermostable. The number and location of Pro residues is also crucial to the stability of protein (Evdokimov *et al*., 2001). Site directed mutagenesis studies with ribonuclease A, where Ala20 was substituted for Pro in the loop region, extremely increased the proteolytic resistance of the enzyme (Markert *et al*., 2001). The proteins are attacked by protease in the regions that are accessible and flexible

Fig. 4: Figure showing the position of Ile (red) and Pro (blue) residues on the modeled protein

such as the coils. The number of Pro residues in wheat germin is 16 i.e.8.0% of total residues (a value very similar that of thermophilic proteins), out of which 13 were present in the region other than helices and strand such as coils (Fig. 4). Presence of Pro in coils might have stabilized and made the coils resistant to be cleaved by proteases.

CONCLUSION

The present *in silico* study describes some important physiochemical, functional and structural properties of wheat germin. Physiochemical and functional analysis reveals that wheat germin is a basic and hydrophobic protein which has one disulphide linkage. Instability index and aliphatic index values for wheat germin are comparable to that found for stable proteins. Highly reliable three- dimensional structure of wheat germin, modelled on closely related structure of barley germin (RMSD 0.26 A^o) is also predicted. Model validation results by five different tools (PROCHECK, Verify 3D, WHAT IF, ERRAT and ProSA) shows the modelled structure to be highly reliable. A careful analysis of the structure reveals the presence of Glu (E^{95}) residue along with three His residues (H^{88}, H^{90} and H^{137}) at ligand binding site. Further, the structural analysis suggests that presence of five Ile residues at β-barrel core and 13 Pro residues in the coil region may be responsible for higher thermal and proteolytic stability of wheat germin. Structural characterization and stability considerations for wheat germin might be exploited in wet lab for site directed mutagenesis studies to improve the stability of enzyme and for biotechnological applications such as for oxalate biosensor development.

ACKNOWLEDGMENT

The author is thankful to Dr. Manoj, Scientist Group IV, IMTECH, Chandigarh and Sh. Rajnikant for helpful guidance and support.

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