Docking of Polyaniine with D-Amino Acid Oxidase of *Rhodosporidium toruloides* and Pig Kidney-An Insight into the Mechanism of Binding for Immobilization in Polyaniine Supports

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**Abstract**

The interaction of Polyaniine (PAni) with *Rhodosporidium toruloides* D-amino acid oxidase (RtDaao) and pig kidney D-aaoo (PkDaao) was studied by bioinformatics approach. The interaction of PAni with RiDaao does not interfere with the substrate binding and hence the D-amino acid oxidase (D-aaoo) activity is unaffected by the ligand. The active site cavity of pig kidney D-aaoo (PkDaao) is comprised of the residues Leu 51, Tyr 224, Tyr 228, Arg 283 and Gly 313. PAni interacts with Leu 51 and Thr 317 of chain G of the PkDaao with interaction energy of -2.5 and -1.09 kcal/mol respectively. D-aaoo was immobilized onto PAni-sodium alginate beads using glutaraldehyde as the crosslinking agent. 1g of the PAni-sodium alginate D-aaoo beads contained 0.093 ml or 0.385 U of the D-aaoo enzyme. The Activity Yield (AY) was calculated as 18.92% as determined by the pyruvate method of detection while, the AY was calculated as 17.3% as determined by the o-PDA method of detection of the beads. The PAni-sodium alginate D-aaoo beads were characterized by Fourier Transform Infrared spectroscopy (FTIR), X-ray Diffraction (XRD), Energy Dispersive X-ray Diffraction (EDX), Thermogravimetric Analysis (TGA) and Scanning Electron Microscopic analysis.

**Keywords:** Polyaniine; D-amino acid oxidase; *Rhodosporidium toruloides*; Docking; Immobilization

**Introduction**

D-amino acid oxidase (D-aaoo) is a FAD-dependent oxidase that catalyzes the oxidation of D-amino acids to their corresponding α-ketoacids with the production of ammonia and hydrogen peroxide. D-aaoo can accomplish distinct regulatory or catalytic functions, therefore have been extensively used for the enzymatic deamination of cephalosporin C to 7-ACA, a key intermediate for the production of semisynthetic cephalosporins [1], preparation of α-keto acids for treating chronic uremia [2], resolution of racemic mixtures of amino acids [3], and the detection and quantification of D-amino acids in biological samples [4]. The three-dimensional crystal structures of pig kidney D-aaoo (PDB ID KIF) and *Rhodosporidium toruloides* D-aaoo (PDB ID COI) were downloaded from Protein Data Bank (http://www.rcsb.org/). The secondary structure of *Rhodosporidium toruloides* D-aaoo (RtDaao) consists of 11a helices and 13β strands and is analogous to that of pig kidney D-aaoo (PkDaao), but RtDaao has three additional short a helices in the head region and a considerably shorter (6 residues as compared to 11 residues in PkDaao) active site loop [5-7]. Also, RtDaao has a high r.m.s deviation of 1.38 Å for 281 superimposable Ca atoms which suggests the evolutionary distance between the two enzymes.

PkJkDaao subunit is divided into two domains, the FAD binding domain with the dinucleotide binding motif or the conserved Rossman fold [8] and the interface domain characterized by a large eight-stranded mixed 13 sheet [5]. The FAD-binding patterns of both enzymes share some similarities while possessing some differences. The RtDaao active site is a cavity comprised of two long antiparallel β-strands bent around an isoalloxazine ring of the flavin and two short antiparallel β-strands near the substrate binding site. In PkJkDaao a loop containing the Tyr224 residue which is involved in substrate/product fixation [9] and interaction between the substrate α-amino group and an active site water molecule is absent in RtDaao and the side chain of Tyr238 is placed in a similar position.

A highly conducting polymer, polyaniine has many applications and interesting features like its controllable electrochemical and electro-optical properties [10,11] and its unique thermal and electrochemical stability [12,13]. Various enzymes like peroxidase [14], glucose oxidase [15] and cholesterol oxidase [16] were immobilized onto polyaniine to develop biosensors. Recently, Lata et al. [17] developed a D-amino acid biosensor based on D-amino acid oxidase/carboxylated multiwalled carbon nanotube/copper nanoparticles/polyalnine modified gold electrode. In the present study, an attempt was made for immobilizing D-aaoo onto polyaniine (PAni) support for future development of biosensors for D-amino acids. For this, the docking of PAni with D-aaoo of both *Rhodosporidium toruloides* and pig kidney was done with a view to study the in silico molecular interaction of PAni with the enzyme. Further, pig kidney D-aaoo was immobilized onto PAni in the form of PAni-sodium alginate beads and the immobilized beads were characterized by Fourier Transform Infrared spectroscopy (FTIR), X-ray Diffraction (XRD), Energy Dispersive X-ray Diffraction (EDX), Thermogravimetric Analysis (TGA), Energy Dispersive X-ray (EDX) and Scanning Electron Microscopy (SEM) analysis. The activity yield (%) of the immobilized beads was also calculated. This study would help in knowing the interactions between PAni and D-aaoo for immobilization of the enzyme in PAni supports which could be exploited for the development of biosensors for D-amino acids.

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Materials and Methods

Chemicals

Pig kidney D-amino acid oxidase (1.4.3.3) from Sigma-Aldrich, India (Product code A5222) was taken for its immobilization onto Polyaniline. Aniline, ammonium persulfate and glutaraldehyde were purchased from Merck, Mumbai, India.

Generation of chemical structures

Two dimensional structure of PAni was downloaded from NCBI Pubchem database [18] and the three dimensional structure was generated using Cambridge Soft ChemOffice 2010 [19]. Energy minimization of the generated chemical structure was done using MM2 force field methods [20] and saved as SYBL mol2 files.

Computation

Molegro Virtual Docker (MVD) was used for the docking studies [21]. MVD is molecular visualization and molecular docking software which is based on a differential evolution algorithm; the solution of the algorithm takes into account the sum of the intermolecular interaction energy between the ligand and the protein and the intramolecular interaction energy of the ligand. The docking energy scoring function is based on the modified piecewise linear potential with new hydrogen bonding and electrostatic terms included. Full description of the algorithm and its reliability compared to other common docking algorithm was explained by Thomsen and Christensen [22].

The three dimensional crystal structure of pig kidney D-aao (PDB ID KIF) [5] and Rhodosporidium toruloides D-aao (PDB ID COI) [7] were retrieved from the Protein Data Bank and imported in the MVD software [21]. The ligand PAni with a molecular weight of 214.26 has 4 each hydrogen bond donors and acceptors (HBD and HBA), 0 rotatable B bonds and a TPSA of 99.7.

Rhodosporidium toruloides D-aao (PDB ID COI): For the docking of RtDaaol, all the 572 water molecules and the 3 ligands were removed from the workspace and a discreet grid covering the protein was created with a sphere of radius of 13 Å. The ligand to be docked, PAni (CID_1621394) was imported into the workspace and the cavity was set at X: 28.70, Y: 128.54, Z: 30.29. The docking algorithm was set at maximum iterations of 1500 with an evolution size of 50 and 5 poses for each turn.

Pig kidney D-aao (PDB ID KIF): For the docking of PkDaaol, all the 294 water molecules and 16 ligands were removed. PkDaaol consists of 8 chains (A-H) and the docking was done for each of the chains separately. A discreet grid covering the protein was created with a sphere of radius 13 Å. The following cavities were created for the 8 chains: Chain A, X:63.09, Y:6.27, Z: 56.78; chain B, X:86.65, Y:48.92, Z: 11.45; chain C, X:170.16, Y:51.59, Z: 16.69; chain D, X:130.60, Y:37.13, Z: 44.61; chain E, X:67.07, Y:44.04, Z: 35.79; chain F, X:101.60, Y:11.45, Z: 53.46; chain G, X:131.30, Y:35.93, Z: 5.78 and chain H, X:145.27, Y:76.33, Z: 33.45. The docking algorithm was set at maximum iterations of 1500 with an evolution size of 50 and 5 poses for each turn.

Polymerization of aniline to form polyaniline (PAni) and confirmation of synthesis by FTIR analysis

Polyaniline (PAni) was synthesized from aniline monomers following the method of Singh et al. [23]. The synthesis of PAni was confirmed by FTIR analysis of the PAni with KBr using a FTIR machine (Nicolet Impact I-410).

Preparation of PAni-sodium alginate beads and immobilization of D-aao onto the beads

A solution of 0.2% PAni dissolved in 0.5% glutaraldehyde solution was mixed with sodium alginate solution so that the final volume of sodium alginate was 3%. Then, D-aao (0.5 Units) was mixed with the above solution and poured drop wise over a stirred solution of 0.2 M CaCl2. The D-aao immobilized beads formed were collected by filtration over a Whatman filter paper and the total wet weight of the beads was measured. Blank beads were also made in the same way, except that they don’t contain the D-aao enzyme. The total wet weight of the blank beads was also recorded.

D-aao assay of PAni-sodium alginate beads (o-phenylenediamine and pyruvate method) and calculation of Activity yield

The D-aao assay (pyruvate method) of the immobilized beads was done according to the method of Khang et al. [24] with some modifications. Briefly, 10 mM of D-alanine dissolved in 0.1 M sodium phosphate buffer, pH 8.0 containing 400 U catalase (HiMedia, India) was reacted with 0.3 g of the PAni-sodium alginate-D-aao beads and the pyruvic acid produced was determined by reacting 100 µl of the sample solution with 40 µl of 0.2% 2,4-dinitrophenylhydrazine (in 2 N HCl) at 37°C for 3 min. Then, 140 µl of 3 N NaOH and 1 ml of deionized water were added for another 5 min reaction. The absorbance at 550 nm was measured to calculate the pyruvic acid concentration based on the calibration curve of pyruvate. One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol of pyruvate per minute as per the assay conditions.

The Activity yield of the immobilized PAni-sodium alginate D-aao beads was determined by comparing the activity of the immobilized enzyme to the activity of the initial enzyme used in the immobilization reaction (equation 1).

\[
\text{Activity yield (}\%\text{)=}\frac{(\text{Activity of immobilized d enzyme (D-aao))/Activity of free D-aao})\times100}
\]

Characterization of the D-aao immobilized beads by FTIR, XRD, TGA, SEM and EDX analysis

The FTIR analysis of the D-aao immobilized beads along with blank beads was performed using dried bead and film pellets with KBr using a FTIR machine (Nicolet Impact I-410). X-ray Diffraction Pattern (XRD) of the D-aao immobilized beads were carried out using Miniflex X-ray diffractometer (Rigaku Corporation, Japan) with scanning mode 2 theta/ theta; scanning type being continuous; X-ray 30 kV/15mA; divergence slit being variable; scattering slit 4.2°; receiving slit 0.3 mm; step 0.02 and using Kb filter from 10°C to 70°C. Blank beads were also taken for the XRD analysis. The Thermogravimetric Analysis (TGA) of the D-aao immobilized beads was done in a TGA-50 analyzer; Shimadzu in the range from 20 to 600°C at 10°C/min. Blank beads was also
taken during the analysis. The Scanning Electron Microscopy (SEM) analysis of the blank and D-aao immobilized beads was done using JOEL model no. JSM-6390LV (Oxford Instrumentation Ltd., Tokyo, Japan). For the analysis, the dried blank and enzyme immobilized beads were sprinkled on the carbon tape and then coated with 30 nm platinum coat using JOEL auto fine coater (model no. JFC-1600; Oxford Instrumentation Ltd., Tokyo, Japan). The SEM was operated at 15-20 KV and under 1 Pascal pressure with the spot size fixed at 34. Atomic compositions of the beads with respect to C, N, O, Na, Cl, Ca were determined using Energy Dispersive X-ray diffraction (EDX, micro-analysis). Samples were visualized under the scanning electron microscope (2,000X-4,000X magnifications) using X-ray source 0–20 KV with EDX software.

Results and Discussion

Docking of PAni with *Rhodospiridium toruloides* D-aao and pig kidney D-aao

Polyaniline docked at the binding sites of RtDaao and PkDaao were ranked based on the top poses score as shown in Table 1. Both bonded and non-bonded interactions were observed between PAni and RtDaao at residues Thr 1179 and Asp 1036 as shown in Figure 1a and 1b. The active site cavity of RtDaao was comprised of the residues Asn 54, Met 213, Tyr 223, Tyr 238, Arg 285, Ser 335, Gln 339 [7]. The residue that appears to play a major role in modulating the substrate preference of yeast Daao was Met 213, which belonged to the hydrophobic pocket of the active site [7]. Thus interaction of PAni does not interfere with the substrate binding and hence the D-amino acid oxidase activity was unaffected by the ligand. Figure 2a-2d and Figure 3a-3d, shows the predicted bonded interactions between PAni and the eight chains (A-H) of PkDaao. Interaction was seen at residues Val 47 (chain C, D, E), Leu 51 (chain G), Gly 198 (chain C, D, E), Gly 200 (chain A, B, F, H), Gly 281 (chain C, D, E) and Thr 317 (chain G). As reported by Mattevi et al [5], the active site cavity of PkDaao was comprised of the residues Leu 51, Tyr 224, Tyr 228, Arg 283 and Gly 313. As seen in Figure 3c, PAni interacted with Leu 51 and Thr 317 of chain G of the PkDaao with interaction energy of -2.5 and -1.09 kcal/mol respectively. Thus a lower binding affinity in case of Leu 51 suggested that the interaction between the ligand and the enzyme is weak. Although Leu 51 forms the active site of the pig kidney enzyme, the weak interaction might not affect the enzyme’s activity with respect to substrate binding.

### FTIR analysis of Polyaniline (PAni)

PAni formed from aniline monomers was confirmed by FTIR analysis (Figure 4). The peaks at 1147 cm⁻¹ were due to vibrations emerging from C-N bond, peak at 1309 cm⁻¹ were due to C-N in quinoid benzoid quinoid sequence while the peaks at 1484-1573 cm⁻¹ represented the C=C vibrations of quinoid benzoid rings [16]. The peaks at 725 and 817 cm⁻¹ represented the stretching and bending vibrations of C-N.

### Immobilization of D-aao onto PAni-sodium alginate beads and calculation of the Activity Yield (AY)

The total weight of PAni-sodium alginate D-aao beads obtained after immobilizing 0.121 ml D-aao (0.5 U), was measured as 1.3 g. Thus, one gram beads contained 0.093 ml or 0.385 U of the enzyme. The D-aao assay of the beads was performed with 0.3 g beads by both the pyruvate detection as well as the o-PDA method as described earlier. According to the pyruvate method of detection, the AY was calculated as 18.92% while, the AY was calculated as 17.3% as determined by the o-PDA method of D-aao assay of the beads.

### Characterization of the D-aao immobilized beads by FTIR, XRD, EDX, TGA and SEM analysis

The FTIR spectra of the PAni-GA-sodium alginate beads (Figure 5a) show the characteristic peaks of alginate at 1052 and 1461 cm⁻¹ representing the asymmetric C-O-C stretch and a symmetric CO₂ᵢ stretch respectively. A significant peak at 3448 cm⁻¹ represents the OH vibration of water. The peaks 1290 and 1452 cm⁻¹ are characteristic of

| S.N. | Protein            | Rerank pose score | H-Bond   | Residue     | Interacting atom name (protein) | Interacting atom ID (ligand) | E Intra | Interaction E (kcal/mol) | Interaction Distance (Å) |
|-----|-------------------|------------------|---------|-------------|-------------------------------|-----------------------------|--------|------------------------|--------------------------|
| 1   | RtDaao            | -55.486          | -1.848  | Thr 1179    | O                             | N (5)                       | -0.546 | -0.823                 | 3.04                     |
| 2   | PkDaao chain A    | -48.559          | -2.5    | Gly 200     | O                             | N (0)                       | -0.547 | -2.0                   | 2.82                     |
| 3   | PkDaao chain B    | -48.232          | -2.37   | Gly 200     | O                             | N (0)                       | -0.547 | -2.37                 | 2.99                     |
| 4   | PkDaao chain C    | -48.129          | -2.29   | Val 47      | O                             | N (0)                       | -0.547 | -1.325                 | 2.67                     |
| 5   | PkDaao chain C    | -48.129          | -2.29   | Gly 198     | O                             | N (5)                       | -0.545 | -0.878                | 3.3                      |
| 6   | PkDaao            | -48.129          | -2.29   | Gly 281     | O                             | N (5)                       | -0.545 | -0.093                | 2.73                     |
| 7   | PkDaao chain D    | -47.922          | -2.54   | Gly 198     | O                             | N (5)                       | -0.546 | -2.07                 | 3.18                     |
| 9   | PkDaao chain E    | -48.459          | -2.05   | Val 47      | O                             | N (0)                       | -0.547 | -2.5                  | 2.74                     |
| 10  | PkDaao chain E    | -47.459          | -1.63   | Val 47      | O                             | N (0)                       | -0.547 | -0.956                | 3.19                     |
| 11  | PkDaao chain F    | -47.981          | -3.59   | Gly 200     | O                             | N (5)                       | -0.546 | -1.88                 | 2.91                     |
| 12  | PkDaao chain G    | -46.238          | -3.59   | Gly 200     | O                             | N (5)                       | -0.546 | -2.5                  | 2.6                      |
| 13  | PkDaao chain H    | -48.299          | -2.5    | Gly 200     | O                             | N (5)                       | -0.546 | -2.5                  | 2.82                     |

Table 1: Molecular docking poses score of PAni (CID_16213194) with RtDaao and PkDaao. *The rerank score is a linear combination of E-inter (steric, Van der Waals, hydrogen bonding, electrostatic) between the ligand and the protein, and E-intra (torsion, sp²-sp₂, hydrogen bonding, Van der Waals, electrostatic) of the ligand weighted by pre-defined coefficients.
the PANi backbone which represent the C-N in the quinoid benzoid sequence of PANi and C=C vibration of the quinoid benzoid ring of PANi respectively. A small peak at 2946 cm\(^{-1}\) was due to the GA crosslinking duplet \(\gamma\)CH\(_2\) [26]. The peak at 1650 cm\(^{-1}\) in the PANi-GA-sodium alginate-D-aao beads represents the carbonyl stretch (amide I band) of the D-aao molecule [27]. In the X-ray diffraction spectra of the PANi-GA-sodium alginate-D-aao beads (Figure 5b), the peaks at 2\(\theta\) (20.2° and 26.45°) can be ascribed to the parallel and perpendicular periodicity of polyaniline [28] while the peaks at 2\(\theta\) (16.7°, 18.6°, 23.2°, 29.3°, 48.35° and 55.75°) may result from the X-ray diffraction of the D-aao enzyme.

The elemental composition analysis of the PANi-GA-sodium alginate-D-aao beads and blank beads with respect to C, N, O, Na, Cl and Ca (Figure 5c) were determined using Energy Dispersive X-ray Diffraction (EDX, micro-analysis). The atomic % of the elements C, N and O of the blank PANi-GA-sodium alginate beads were lower i.e. 3.52%, 10.23% and 1.63% respectively than that of the PANi-GA-sodium alginate-D-aao beads.

The thermogravimetric analysis of blank PANi-GA-sodium alginate beads and PANi-GA-sodium alginate-D-aao beads is shown in Figure 5d. The D-aao immobilized beads were found to be thermally stable as compared to the blank beads. The initial weight loss at 179°C observed for both blank as well as the PANi-GA-sodium alginate-D-aao beads can be attributed to residual water retained after drying. At 279°C, there was a drop in the weight of the beads wherein the blank beads showed lower thermal stability than the PANi-GA-sodium alginate-D-aao beads. The major weight loss occurring at 510°C was ascribed to the thermal decomposition.

Figure 6 shows the scanning electron micrographs obtained for the blank and PANi-GA-sodium alginate-D-aao beads. The SEM image of the blank beads showed the presence of spongy and porous morphology...
Figure 5: (a) FTIR spectra of the PAni-GA-sodium alginate beads (blank beads) and PAni-GA-sodium alginate-D-aao beads (D-aao immobilized beads); (b) X-ray diffraction spectra of the PAni-GA-sodium alginate (blank beads) and PAni-GA-sodium alginate-D-aao beads (enzyme loaded beads); (c) Energy Dispersive X-ray diffraction analysis with respect to different atoms, of PAni-GA-sodium alginate (blank beads) and PAni-GA-sodium alginate-D-aao beads (D-aao beads); (d) The thermogravimetric analysis of blank PAni-GA-sodium alginate beads and PAni-GA-sodium alginate-D-aao beads.

Figure 6: Scanning electron micrographs obtained for the blank PAni-GA-sodium alginate and PAni-GA-sodium alginate-D-aao beads. (a), (b), (c) are the SEM images for the blank beads at different magnifications while (d), (e), (f) are the SEM images of the D-aao immobilized beads at different magnifications.
of polyaniline (Figure 6a–6c), which might be helpful in the entrapment of the enzyme molecules. Upon immobilization of the D-aaO onto the PAni-GA-sodium alginate beads, the globular structural morphology of the enzyme could be clearly seen (Figure 6d–6f).

Conclusion

Immobilization of D-aaO is important for the development of biosensors as the use of the enzyme in free form is restricted by the poor thermal stability and the sensitivity to hydrogen peroxide inactivation [29]. Applications of D-aaO in immobilized form can offer several advantages for industrial applications including repeated use, ease of separation of reaction products, improvement of enzyme stability and so on [30]. From the present study it can be concluded that Polyaniline interacts with negative and least interaction at the active site of the Rhodosporidium toruloides so on [30]. From the present study it can be concluded that Polyaniline interacts with negative and least interaction at the active site of the Rhodosporidium toruloides and pig kidney enzyme respectively thereby un-affecting the enzyme’s activity upon immobilization. Thus the immobilization of D-aaO in Polyaniline supports is possible which may be further exploited for the future development of D-aaO biosensor based on Polyaniline supports for determination of D-amino acids.

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