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# Studies on binding modes between salivary amylase-food dyes and effect of Copper ions: molecular docking approach

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**ABSTRACT**

*The interaction between metal ions and the oral environment is a major subject matter in dental research. The saliva acts as an electrolyte solution for different dental alloys. Saliva is expected to be of significance for the perception of food stimuli in the mouth. Mixing the food with saliva, including breakdown and dilution, is considered to be of large importance for semi-solids as these products are masticated without chewing, Saliva contains a range of enzymes that are susceptible to proteolysis. α-Amylase, present in large concentrations in whole saliva (WS). Starch is hydrolyzed to glucose, maltose and dextrin by α- and β-amylases and other related enzyme. In the other hand the affinity interactions between pigments and saliva were influenced by some factors, such as functional groups, molecular weight of pigments, temperatures, pH values, and salt concentrations. All these factors suggest that the complex of pigments may be difficult to remove from the WS film. The aim of this study was to test the effect of metal ions (copper) released from dental amalgam on the α-amylase activity.*

**Keywords:** Food dyes, Copper(II), α-amylase activity, Molecular Docking, Interaction.

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## INTRODUCTION

Dental amalgam, an alloy formed by mercury with another metal or metals, is one of the oldest materials used in restorative dentistry, and still is one of the most commonly employed. Several metal ions are constantly released from amalgam by electrochemical reactions [1-3].

Part of these ions are formed the nature complexes with some food dyes. Azo dyes account for the majority of all dyestuffs, produced because they are extensively used in the textile, paper, food colorants, leather, cosmetics and pharmaceutical industries [4]. Azo dyes make up approximately 70% of all dyestuffs used worldwide by weight [5], making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment [6-8]. Azo dyes absorb light in the visible spectrum due to their chemical structure, which is characterized by one or more azo groups (–N=N–) [9].

The complexes formed by $Cu^{2+}$ with some food dyes (amaranth **,** ponceau 4R**,** and sunset yellow**)** has been studied spectrophotometrically under varied conditions [10],indigo carmine [11], tartrazine [12] .

In the other hand the properties and behaviour of such chemical systems in the oral cavity are poorly known, the terms ‘‘whole saliva’’, ‘‘mixed saliva’’ and ‘‘oral fluid’’ are used to describe the combined fluids present in the oral cavity. This fluid is mainly composed of water (99.5%), proteins (0.3%) and inorganic and trace substances (0.2%) [13-15] .The proteins in saliva (1–2 mg/ml) are mainly constituted by glycoproteins [13] enzymes (e.g., α-amylase,

carbonic anhydrase), immunoglobulins, and a wide range of peptides (cystatins, statherin, histatins, proline-rich proteins) with antimicrobial activities [13,15-17].

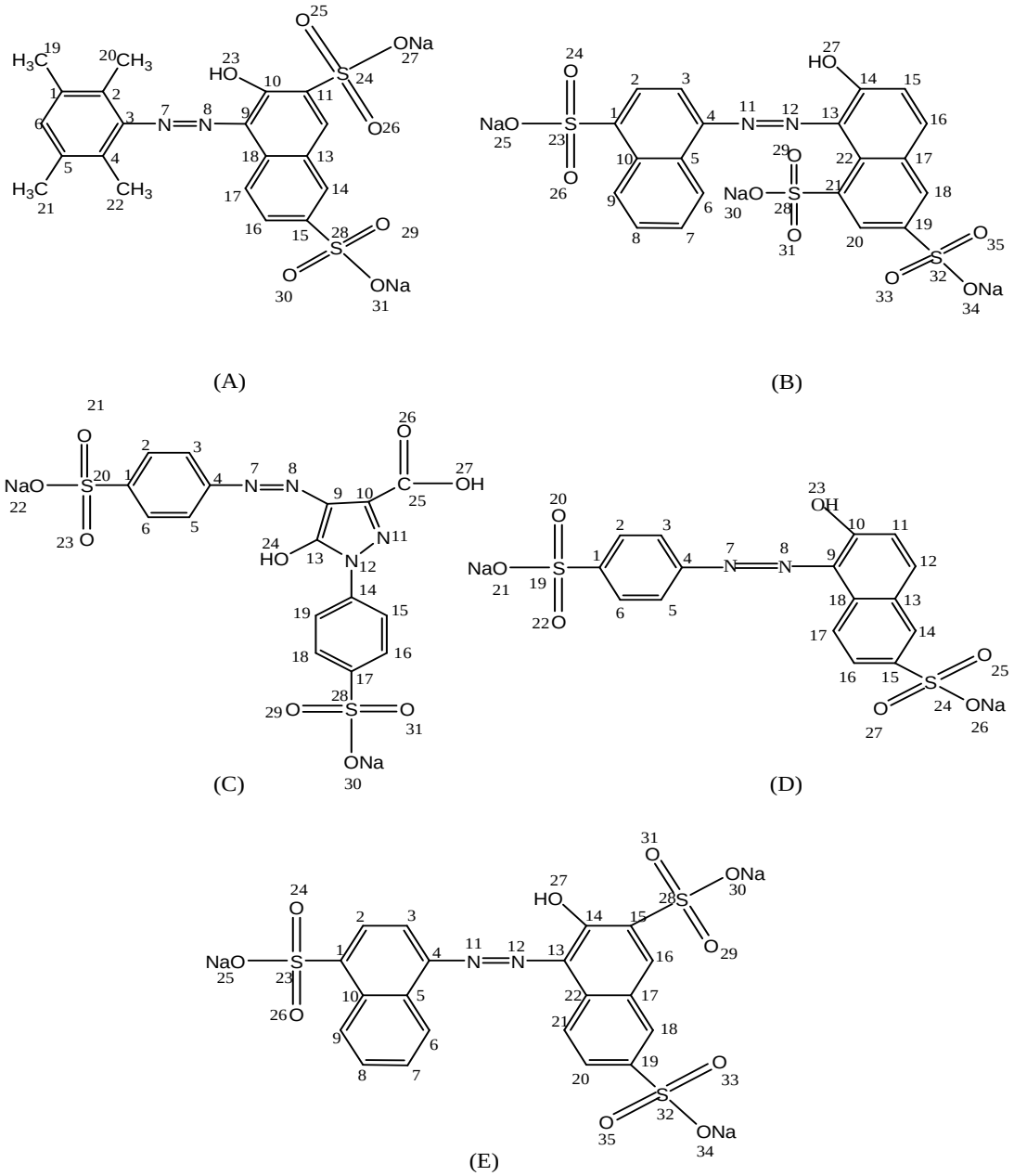


**Fig.1. (A): Ponceau 4R, (B): Cochineal red A, E 124, (C): Tartrazine, E 102, (D): Sunset Yellow: E110, (E): Amara**

Although the oral cavity is the initial site exposed to food pigments [18], little information exists on the molecular dynamics of the response of interactions between natural pigments and proteins [19-22], particularly the mixed salivary proteins.

The affinity interactions between pigments and saliva were influenced by some factors, such as functional groups, molecular weight of pigments, temperatures, pH values, and salt concentrations. All these results suggest that the complex of pigments may be difficult to remove from the whole saliva (WS) film [23].

Saliva contains a range of enzymes that are susceptible to proteolysis. α-Amylase, present in large concentrations in WS [24]. Starch is hydrolyzed to glucose, maltose and dextrin by α- and β-amylases and other related enzyme [25]. The interaction between the dye (Cibacron Blue F3GA (CB)) ligand and α-Amylase can be by complex combination of electrostatic, hydrophobic, and hydrogen bonding [26].

In this study, computations on the interactions at the active site of α-Amylase were carried out for five ligands. All these ligands have shown to be competitive or non competitive inhibitors for the α-Amylase activity.

The availability of several co-crystallized structures for α-Amylase with different inhibitors makes it possible to apply a molecular docking protocol to explore the enzyme-inhibitor interactions.

The study also focuses on the comparison between the inhibitory potentials of these five free food dyes on α-Amylase and the inhibitory potentials of these five complexed food dyes on α-Amylase. Also we examined the effects of $Cu^{+2}$ on the activity of the major α-amylase.

## MATERIALS AND METHODS

### 2.1. Ligands structure

A series of food dyes ligands (figure 1) as α-amylase inhibitor is considered in this study. For the geometry optimization, the ligand structures were prepared by use of the MM+ force-field (calculations in vacuo, bond dipole option for electrostatics, Polak–Ribiere algorithm, and RMS gradient of 0.01 kcal/Å mol) as implemented in HyperChem7.0 [27]. The most stable conformer was fully optimized with AM1 semi-empirical molecular orbital calculations encoded in the Gaussian03 [28]. Conformer energy of compounds was calculated.

### 2.2.PDB entries used in search

The three dimentional coordinates of α-amylase (PDB ID: 1XV8) [29] were obtained through the Protein Data Bank. (www.rcsb.org/pdb) [30]. Waters, cofactors and originally bounded ligands for α-amylase were removed from the docking procedure (figure 2).

We used the molecular docking to try to understand the interactions between:
1/α-amylase and free five food dyes.
2/α-amylase and complexed food dyes.
Were carried out employing the Molegro Virtual Docker (MVD 2011) [31-33] program, graphical-automatic software (http://molegro.com/mvd-product.php).

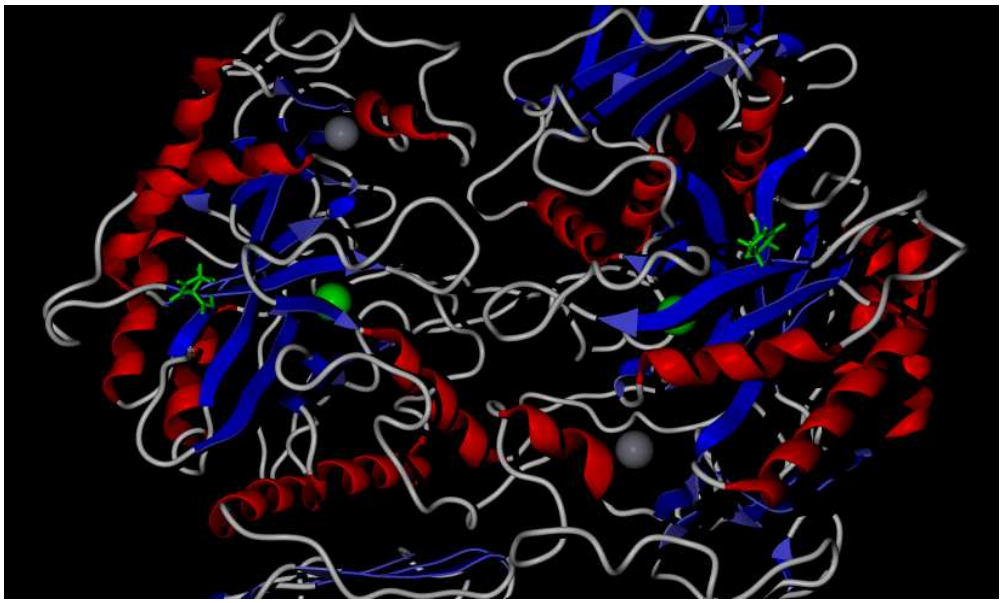

**Fig. 2. The dimerous form of α-amylase**

## 1. DOCKING CALCULATION PROCEDURES

The goal of this paper was to develop methodology for understanding mode of reductive activity of azo dyes. This methodology will be used in future studies for selection of compounds for focused screening libraries.

Two assumptions were central to this approach: 1) competitif antagonism , 2) non competitif antagonism.

Several studies have shown that azo dyes inhibit enzyme activity [34]. One study reported that the enzyme activity of amylase was reduced by 66% with tartrazine and 64% with erythrosine.

In order to test the validity of assumptions, we docked the free and complexed ligands into the active site of α-amylase crystal structure (1XV8.pdb) as well as in the other that may be influence on the enzyme activity indirectly. Molegro Virtual Docker (MVD) 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. It could be accurately forecast the active sites of protein molecules based ligands. MVD is a precise semi-flexible molecular docking program. By inceaseing the qualifications, the recognition accuracy of bonding models is enhanced. Compared with the other dock softwares, the accuracy of MVD is higher. (MVD: 87%, Glide: 82%, Surflex: 75%, FlexX: 58%) [35,36].

**MolDock Algorithm**

The MolDock scoring function (MolDock Score) used by Molegro Virtual Docker program is derived from the PLP (Piecewise Linear Potential. The MolDock score uses a PLP to approximate the steric energy), (approximate binding energies between protein and ligand, generally expressed in kcal/mol) .Scoring functions originally proposed By Gehlhaar et al. and later extended by Yang et al. The scoring Function was further improved to include new charge schemes and hydrogen bonding term [37].

MolDock [35] is an implementation of Evolutionary algorithm (EA), focused on molecular docking simulations [38]. Computational approximations of an evolution process, called genetic operators, are applied to simulate the permanence of the most favorable features. In a sample space, where there is a problem or a search routine and many different possible solutions (candidates), each option is ranked based on a set of parameters (scoring function, or fitness function), and only the best ranked solutions are kept for the next iteration. This process is repeated until an optimal solution can be found.

The program MolDock makes use of a slight variation of the EA, which is called guided differential evolution algorithm. This methodology is based on an EA modification called differential evolution (DE), which brings a different method to choose and alter candidate solutions (individuals). The major original idea in DE is to generate offspring from a weighted difference of parent solutions. The DE works as follows. In the first step, all individuals are initialized and evaluated according to the fitness function. Afterward, the following process will be carried out if the termination condition is not satisfied. For each individual in the population, an offspring is created by adding a weighted difference of the parent solutions, which are randomly chosen from the population. After that the offspring replaces the parent, if and only if it is fitter. Otherwise, the parent survives and is passed on to the next generation (iteration of the algorithm). The termination condition is reached when the current number of fitness (energy) evaluations performed exceeded the maximum number of evaluations allowed (max evaluations parameter setting). Furthermore, early termination was permitted if the variance of the population was below a certain threshold (0.01 here). Moreover guided differential evolution employs a cavity prediction algorithm to limit predicted conformations (poses) during the search procedure. More specifically, if a candidate solution is placed outside the cavity, it is translated so that a randomly chosen ligand atom will be located within the region spanned by the cavity. (Figure 3) shows the cavities predicted by MolDock. Obviously this strategy is only employed if a cavity has been found. If no cavities are reported, the search process does not limit the candidate solutions.

To obtain better potential binding sites in the α-amylase (PDB ID: 1XV8), a maximum of five cavities was detected using default parameters. The volume and surface area details were given as (Table 1). The volume of cavity 1 was found to be highest than the other cavities, also we found that the reference ligand of α-amylase is fixed in cavity 2. Out of the detected cavities, cavity 1 and 2 was selected for further studies (figure 3). The chosen cavity was further refined using side chain minimization by selection of an add-visible option set at a maximum of 10,000 steps per residue and at a maximum of 10,000 global steps. The grid resolution was 0.30 Å; the max iterations were 1,500; the max population size was 50 and the energy threshold was 100.

**Table 1. Chemical properties of our cavities**

| Cavities | Volume Å$^3$ | Surface Å$^2$ |
|---|---|---|
| Cavity 1 | 141.312 | 411.84 |
| Cavity 2 | 42.496 | 170.24 |
| Cavity 3 | 34.304 | 142.08 |
| Cavity 4 | 32.256 | 154.92 |
| Cavity 5 | 24.064 | 92.16 |

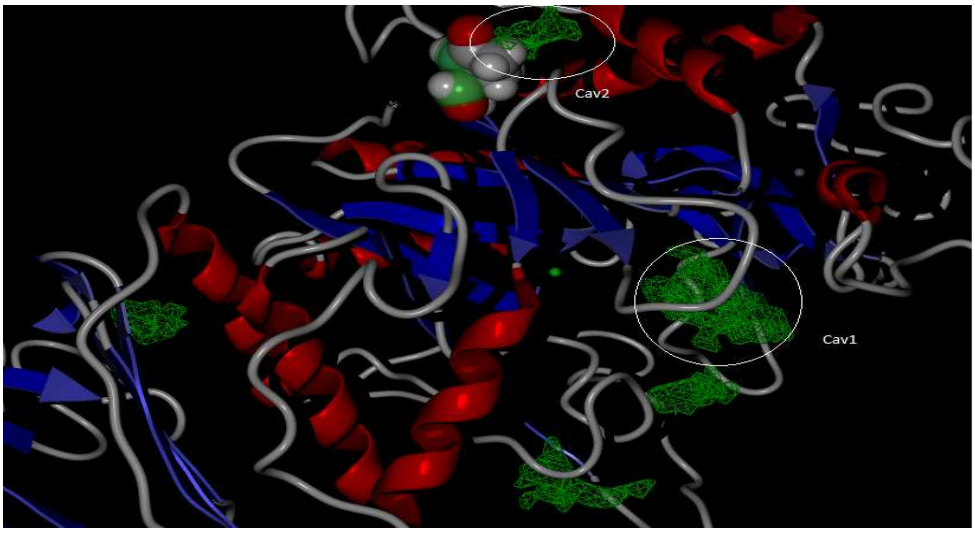


**Fig.3. Graphical interface with the cavities (cavity 1 and cavity 2 indicated by ellipses) identified by MolDock.**

## RESULTS AND DISCUSSION

### 4.1 Chemical properties of ligands:

In this in silico analysis, the basic goal is to analyze the interaction between the α-amylase with a number of food dyes. Although only tartrazine was reported to have reductive activity against α-amylase , it is also a part of this work to assess such property in silico for other food dyes, for which the activity against α-amylase has not been experimentally evaluated out.

Table 2: summarizes the chemical properties of compounds used in docking experiments. The conformer energy value of (B) was found to be less than the other ligands, which explain: (B) has the most stable structure. In the other hand topological polar surface area for the dataset were greater than 966.20 $Å^2$ and lesser than 1179.94 $Å^2$ indicating a high possibility of complete absorption [39].

**Table 2. Chemical properties of our compounds**

| Compound | Conformer energy (kcal/mol) | Volume $Å^3$ | Surface $Å^2$ |
|---|---|---|---|
| A | -188.381 | 1179.94 | 679.56 |
| B | -251.823 | 1166.72 | 656.32 |
| C | -159.076 | 1160.52 | 705.05 |
| D | -165.351 | 1018.09 | 619.10 |
| E | -257.471 | 966.20 | 580.94 |

### 1.2 Molecular Docking Analysis

***a. Part 1: Results: Interaction of free ligands with α-amylase in the both cavities***

To gain more details of the possible binding mode of the compounds and the interacting residues of the α-amylase enzyme (PDB ID: 1XV8), we performed molecular docking analysis. The molecules were docked into the activator binding site using the crystal structure of (Protein Data Bank code: PDB ID: 1XV8).

**Table 3. Docking results of free ligands with α-amylase in the both cavities.**

| | Cavity 1 | | | Cavity 2 | | |
|---|---|---|---|---|---|---|
| | MolDockScore[a] | Interaction[b] | H-bond | MolDock Score[a] | Interaction[b] | H-bond |
| A | -115.227 | -127.464 | -5.234 | -120.863 | -132.882 | -4.373 |
| B | -125.352 | -129.437 | -3.030 | -137.174 | -153.394 | -7.089 |
| C | -140.226 | -146.618 | -15.272 | -142.101 | -151.698 | -10.231 |
| D | -103.239 | -116.030 | -5.585 | -126.009 | -137.292 | -7.410 |
| E | -117.944 | -134.071 | -3.235 | -148.422 | -161.116 | -10.878 |

[a] *MolDock score calculated by summing the external ligand interaction (protein–ligand interaction) and internal ligand interaction score using Virtual Molecular Viewer 1.2.0.*

[b] *The total interaction energy between the pose and the target molecules(s).*

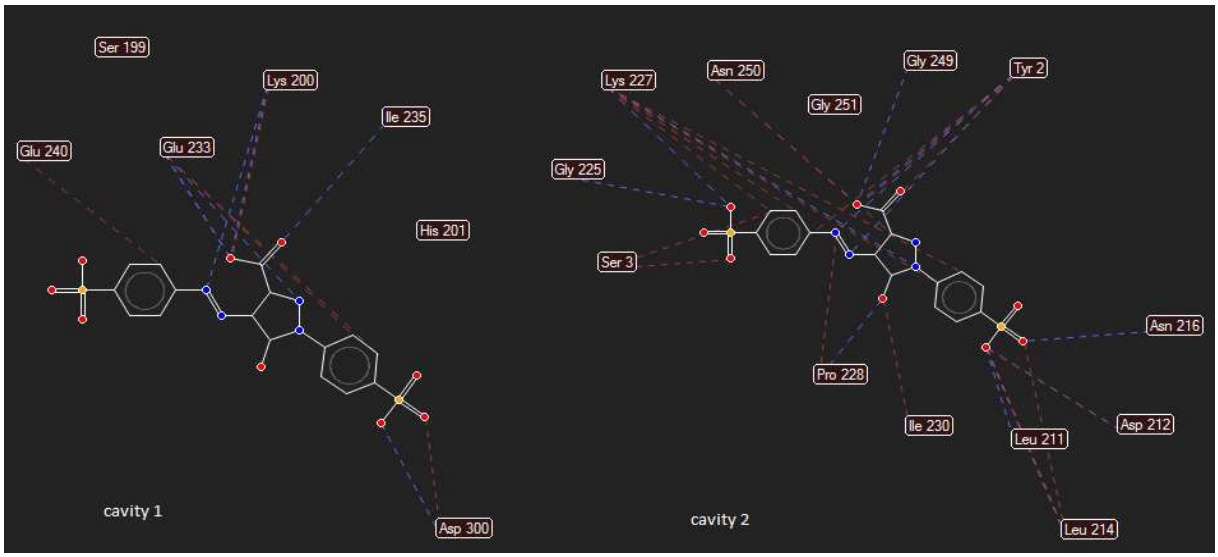


**Fig.4. Predicted protein-ligand contacts of tartrazine in both cavities (------: H-bond interactions, ------: steric interactions).**

The free ligands-receptors, and their corresponding docking results are listed in Table 3, and receptor-ligand interactions demonstrated in Figure 4. In these docking experiments, all selected ligands were able to bind with α-amylase in both cavities. In cavity 2 Amaranth (E) showed the highest binding affinity (MolDockScore kcal/mol) at -117.944, followed by Tartrazine (C), Cochineal red A (B), Sunset Yellow (D) and Ponceau 4R (A) respectively. A similar pattern of binding affinity was found for ligands in cavity 1, except for Amaranth (E). Tartrazine (C) had the highest binding affinity at -140.226 kcal/mol followed by Cochineal red A (B), Amaranth (E), Cochineal red A (B), and Ponceau 4R (A) respectively.

If we compared between the two cavities, it was noted that tartrazine for example (figure 4) in the cavity 2 establish multiple interactions enzyme-substrate, which gives it great stability. For these reasons, we can deduce that cavity 2 is more favorable than cavity 1. These give us an idea on the mechanism of inhibition the azo dyes against α-amylase.
All these results support an assumption 1(previously specified above), that azo dyes play a crucial role as competitive inhibitor, but without ignored the second assumption since we found a quite important affinity of these inhibitors in a cavity 1.

The models revealed the possible binding orientation when ligands were docked into the empty α-amylase enzyme and also showed that ligands were anchored to the binding pocket via similar fashion (figure 5).

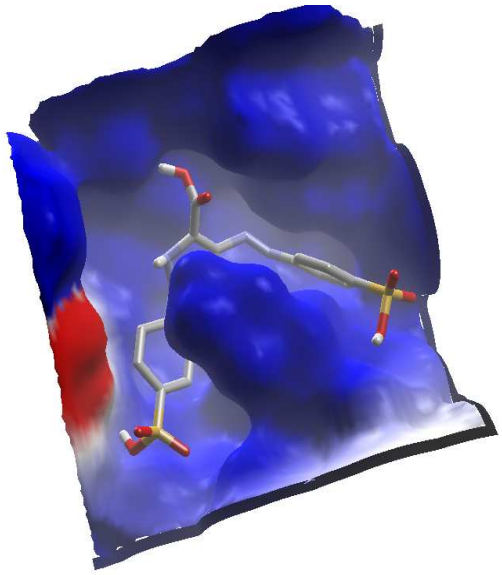

**Fig. 5. Tartrazine is anchored to the binding pocket.**

The hydroxyl and amino groups of ligands were hydrogen bonded to the backbone amide proton and the carbonyls of α-amylase. They could also be involved in additional hydrogen bonding with the side chain of polar amino acid of α-amylase enzyme. All the compounds showed negative MolDock score values and most of them formed hydrogen bonds (up to ~3.2 Å) with the protein residues.
Tartrazine interacted with the α-amylase by forming hydrogen bonds as shown in (Figure 6). The hydroxyl groups $OH_{22}$, $OH_{27}$ and $OH_{30}$ of tartrazine were hydrogen bonded to the backbone carbonyl group of Asp-212, Leu-214 and Tyr-2 and Gly-225 respectively

Therefore, in general, both hydroxyl and amino groups of these molecules are important for their interaction with α-amylase.

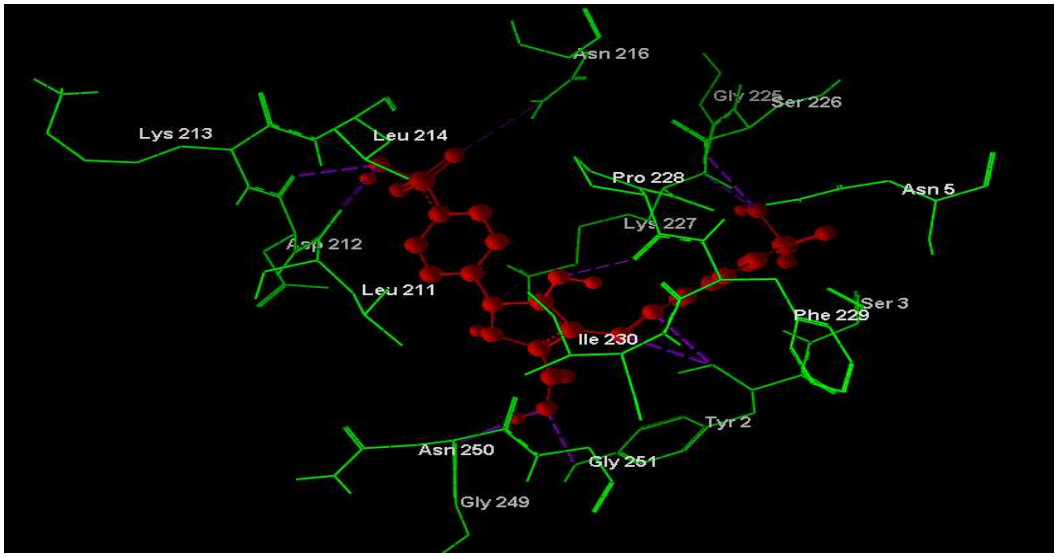


**Fig.6.The binding mode between selected ligand (C) with binding site of α-amylase. Key residues and hydrogen bonds were labeled.**

Table 4 showed the exact hydrogen bond length between the selected compound **(C)** and the keys residues of α-amylase.

**Table 4. Hydrogen bond lengths**

| groups | $OH_{22}$ | | | $O_{21}$ | $OH_{30}$ | | $_7N=N_8$ | $OH_{27}$ | | $OH_{24}$ | $N_{11}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| residues | Asp212 | Leu211 | Leu214 | Asn216 | Gly225 | Ser226 | Tyr2 | Tyr2 | Gly249 | Lys227 | Pro228 |
| Length ( Å) | **2.57** | **3.04** | **2.88** | **3.39** | **2.96** | **3.06** | **2.65**<br>**2.77** | **2.77** | **3.03** | **3.44** | **2.41** |

***b. Part 2: Results: Interaction of free and complexed ligands with α-amylase in cavity2***

The five complexed ligands could be docked on the α-amylase, with energies of interaction per subunit ranging between −87.564 and −122.503 $kJ.mol^{-1}$ (Figure 7).

Figure 8: showed the change interactions between free and complexed Sunset Yellow with the residues of α-amylase.

On docking complexed Sunset Yellow for example is showing bad binding to α-amylase enzymes suggesting that its mechanism of inhibition might be changing in presence copper ions. It was found that the copper changes the conformation of the dye and subsequently the interactions are modified. We note that there appear a new copper-Ile230 interaction and the disappearance of other H-bond interaction, which explains the destabilization of the enzyme-substrate complex by the existence of copper.

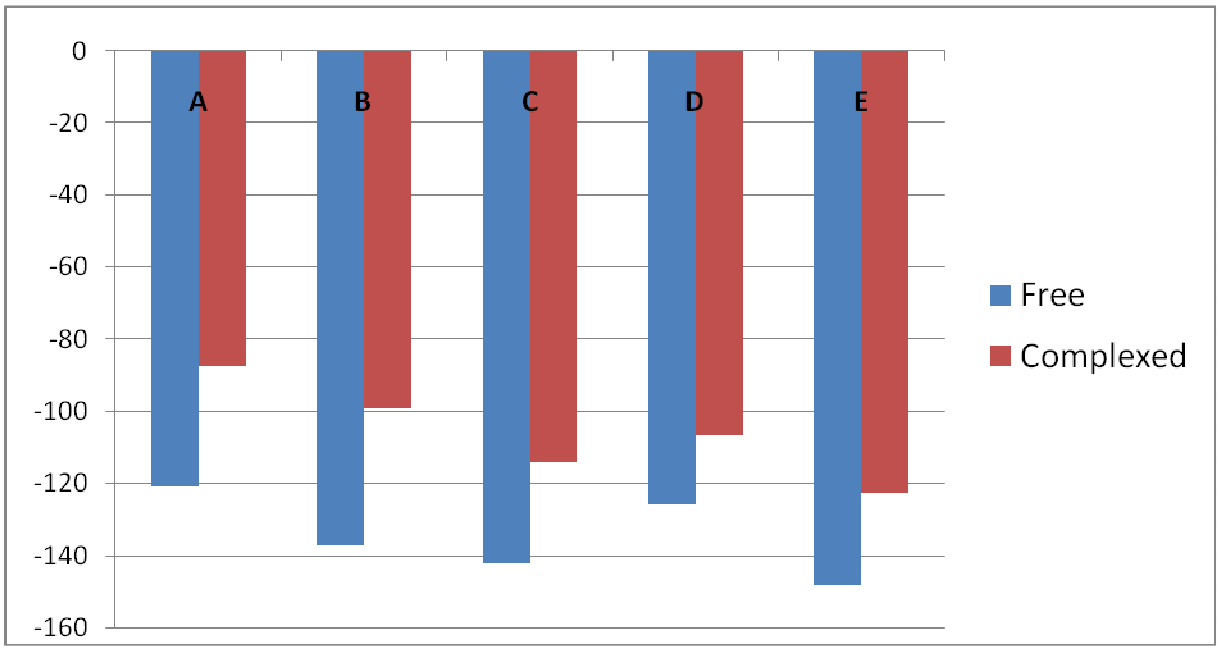


**Fig.7. Graphical representations of MolDock Score values of free and complexed ligand with α-amylase in cavity 2.**

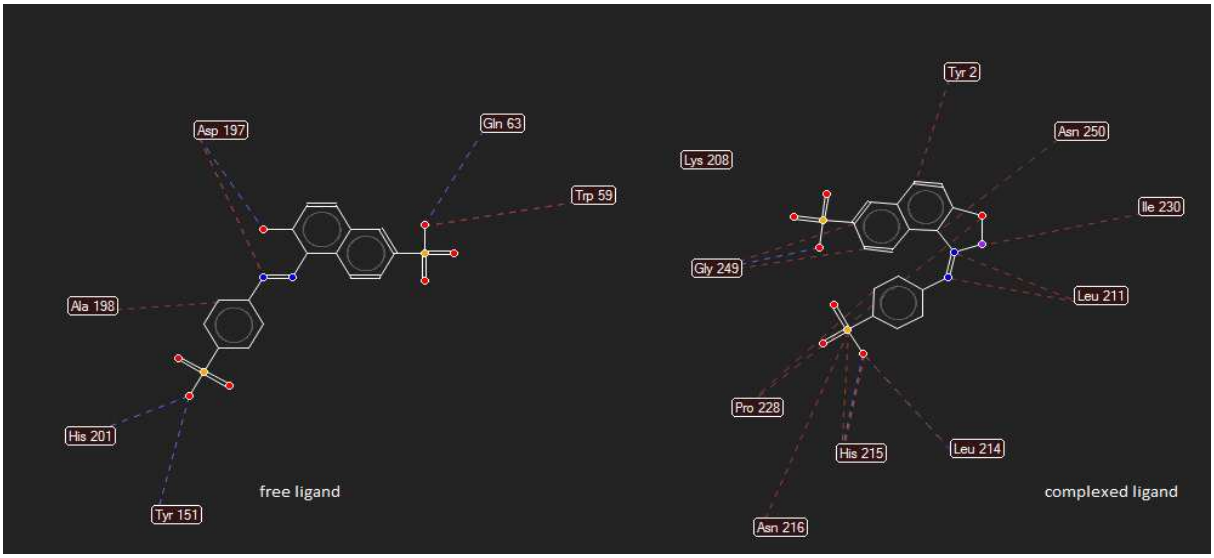


**Fig.8. Predicted protein-ligand contacts of Sunset Yellow in cavity 2 (------: H-bond interactions, ------: steric interactions).**

## CONCLUSION

The results presented in this paper show that, α-amylase is active to break down starch macromolecules into dextrins, with sufficient hydrolysis occurring to make the products soluble and not susceptible to gelling upon cooling. Specifically, α-amylase EC 3.2.1.1. (1,4, α-D-glucan glucanohydrolase) catalyses the hydrolysis of α-1,4 glucosidic linkages in polysaccharides of three or more α-1,4 linked D-glucose units to produce maltose and larger oligosaccharides [40]. There are several evidences indicating that α-amylase play an important role in oral cavity, but copper released from dental amalgam can be directly affect host responses by inhibiting α-amylase activity in presence the food dyes. Also there are a group of compounds such as foods and drugs containing azo function which show good binding affinities with the α-amylase enzyme, why we recommend to avoid using α-amylase with azo dyes and especially for people who have dental amalgams (for example MAXILASE®). In the last our findings show that the activity of oral salivary (α-amylase) may be modulated by metal ions present in the oral environment.

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