ISSN:1991-8178

Australian Journal of Basic and Applied Sciences

Journal home page: www.ajbasweb.com

Computational Investigation of the Monomer Insulin-β-CD Complex for the New Insulin Formulation

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ARTICLE INFO

Article history:
Received 12 March 2015
Accepted 28 April 2015
Available online 2 May 2015

Keywords:
Insulin, β-CD, Computational chemistry, Docking, Molecular dynamics simulation

ABSTRACT

The complexation of insulin with beta cyclodextrin (β-CD) would be to improve absorptive and stability of the formulation insulin. This paper reports our investigation on the interaction between insulin with several β-CDs at the molecular level using the combined docking and molecular dynamics simulation methods. The present study was aimed at finding the best possible ratio of β-CD to insulin, its most stable conformation as well as identifying the type of forces responsible for the stabilization of the complex. A series of 90 ns molecular dynamics simulations were conducted using NPT ensemble to further verify the results obtained by multiple molecular docking approach. Our results show that the most stable conformation obtained was the one insulin to three β-CD ratios, which is stabilized mostly by hydrophobic interactions as well as hydrogen bonds formations. MD analysis of the insulin-β-CD complex system reported a good interaction between insulin and β-CD with the root mean square deviation values of the insulin in complex system stabilized at 80 ns was 0.65 ±0.05 nm. RMSF profiles of the complex system also shows the flexibility of Asn21, Phe22, Val23 and Asn24 residues which are involved in the hydrogen bonding and hydrophobic interactions in docking calculations. The theoretical results indicated the presence of significant interactions between insulin and β-CD which could help in the new insulin formulation.

To Cite This Article: Erma Fatiha Muhammad and Rohana Adnan., Computational Investigation of the Monomer Insulin-β-CD Complex or the New Insulin Formulation. ***Aust. J. Basic & Appl. Sci.,*** *9(17): 7-13, 2015*

INTRODUCTION

The conventional method of insulin administration to diabetic patients has many disadvantages such as common infections, skin bulges, pain due to subcutaneous injections, and stress due to long-term use (Zhang *et al*., 2010). Due to this reason, research to deliver insulin by alternative routes has gained much attention. The oral route has been recognized as the natural and safest route for drug delivery. In order to formulate the insulin for successful oral delivery into the human body, some major problems such as the degradation of insulin by gastrointestinal enzymes, and also poor permeability of insulin across the intestinal epithelium due to its high molecular weight and lack of lipholiphicity (Hosny *et al*., 2002) have to be overcome. Thus, to develop a method of administering insulin in which it is safe with adequate bioavailability and enhance absorption is of immerse importance. Due to the advanced technology in the field of molecular biology, scientists have come out with various approaches to produce an oral delivery insulin such as the chemical modification (Ashada *et al*., 1995), enzyme inhibitors and penetration enhancers (Liu *et al*., 2003) by incorporating insulin into carriers such as hydrogels (Nakamura *et al*., 2014), liposomes (Chaudhary and Labhasetwar, 1994), erythrocytes (Al-Achi and Greenwood, 1998), nanospheres (Damge *et al*., 1997), and nanocubicles (Chung *et al*., 2004). However, these methods have some limitations and disadvantageous such the poor absorption and solubility of insulin into the living system. It has also been proposed that the oral insulin barriers could also be solved via complexing insulin with cyclodextrins (CDs). The complexing of insulin with β-CD could be one of the ways to improve the properties of insulin.

Cyclodextrins (CDs) are oligosaccharides most commonly formed by six, seven and eight glucopyranose units, named as α-CD, β-CD and γ-CD, respectively. Due to its unique properties, a cone-shaped structure with an outer hydrophilic surface and hydrophobic central cavity (Li *et al*., 2007; Loftson *et al*., 2005) (Fig. 1), β-CD is important as it may assist in enhancing the insulin stability and absorption by complexing with insulin.

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This complexation might drastically reduce their state of aggregation in solutions whereby the hydrophobic side chains in the insulin molecule can penetrate into the non-polar CD cavity, leading to the formation of non-covalent inclusion complexes (Irie and Uekama, 1999).

Computational chemistry is a multidisciplinary field of science which could be applied to compliment many areas of sciences including physics, biology, chemistry, mathematics, engineering, as well as in pharmaceutical sciences. The computational methods used today are results from the significant methodological development and advancement in computing power in the recent decades. Computer methods for biological systems are widely used to understand and predict the properties and behavior of systems at the molecular level. The theoretical principles behind this application include the theory of quantum mechanics, molecular mechanics, molecular docking, and molecular dynamics simulation. In recent years, several reports involving the computational study of insulin include the dissociation of the insulin-phenol complex (Kru, 2003), binding of glucose to insulin (Falconi *et al*., 2001; Zoete *et al*., 2004), dynamic behavior of monomer and dimer insulin (Zoete *et al*., 2004), association of glycoprotein structure with B chain human insulin (Stavrakoudis, 2011), insulin stability on graphene (Liang *et al*., 2009), flexibility of B chain human insulin (Legge *et al*., 2006), structure and stability of dimer insulin (Falconi *et al*., 2001), insulin interaction with boron-nitride and functionalized nanosheets graphene (Atabay *et al*., 2014), conformational flexibility of insulin analogs (Ksenofontova and Stefanov, 2013), simulation of the protection of insulin by trehalose (Li *et al*., 2014), and the aggregation and rate of release of insulin (Berhanu and Masunov, 2012).

Herein, we use multiple molecular docking approaches to dock β-CD into the binding site of insulin and understand the conformational change in the structures of insulin-β-CD complex by considering all the possible numbers of β-CD and its binding modes to insulin. To further explore the stability of the complex, structural analysis and dynamics behavior of insulin-cyclodextrin complex in aqueous solution using a 90 ns molecular dynamics simulation was conducted. These computational details could contribute to better understanding of the properties of insulin-β-CD complex, but may also provide potential clues to the experimental researchers for the future development of insulin oral delivery formulation.

Computational Method:

Preparation of receptor and ligands:

The x-ray crystal structure of human insulin (PDB ID: 1BEN) was retrieved from Protein Data Bank (www.rcsb.org), while the structure of β-CD was based from its crystallographic geometry (Alexander, 2002). Since the crystallization form of insulin was formed in dimer (consists of A, B, A' and B' chains), the A' and B' chains of insulin which was located in the original PDB file was removed to get the formation of monomer insulin which contains only of A and B chains. Water molecules were then removed and hydrogen atoms were added to the monomer insulin structure. A single molecule β-CD structure was geometrically optimized using a semi-empirical, PM3 method (Steward, 1989) which is implemented in the Gaussian 2003 program (Frisch *et al*., 2004). The optimized single β-CD structure was then used as ligand in all docking process.

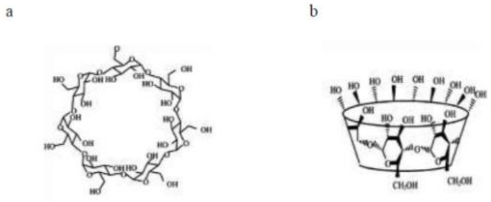

Fig. 1: Molecular structure of (a) top view and (b) side view of β-CD.

Molecular docking using AutoDock4.2:

To find the favorable binding sites between insulin and β-CD, multiple molecular docking was performed with the program AutoDock v4.2 (Morris *et al*., 1998; Morris *et al*., 2009). The multiple molecular docking methods performed consisted of several cycles aimed to find the maximum number of β-CDs which could be bound to the insulin. Lamarckian genetic algorithm search method was used in this study. A hundred independent docking calculations were performed with a maximum of 2.5 × 107 energy evaluations and 100 complex structures obtained for each docking process. These 100 docked structures were clustered according to a root mean square deviation (RMSD) of 2.0 Å and were arranged according to the binding energy. All rotational bonds in β-CD were set free, while the ones of insulin were held rigid. In each docking process, Gasteiger charges were computed for insulin and β-CD structures using the program AutoDockTools (ADT).

The grid maps were prepared using a 126×126×126 Å grid box with the distance between two grid points set at 0.375 Å centering on the insulin. For each docking process, we selected the most stable conformation by determining the binding energy of overall hundred structures. The lowest binding free energy conformation at that binding site was used as an initial receptor in the next docking process. Several docking processes were run using similar methods until there were no more β-CD molecules that could attach at any site of insulin. Each conformation of each docking process which corresponded to the lowest binding energy was selected to be analyzed. As a result, we obtained the

best conformation as well as the possible binding sites between insulin and β-CD and, thus, explained the presence of hydrogen bonds and hydrophobic interaction using the LigPlot (Laskowski and Swindells, 2011) program docking results. The topology of monomer insulin was created using GROMACS based on the GROMACS' database, while the topology of β-CD structures were generated using the PRODRG beta server (Schüttelkopf & van Aalten, 2004). Initially, the insulin and the insulin-β-CD complex were solvated with 7521 of a simple point charge (SPC) water molecules (Berendsen *et al*., 1981) in a 238.163 nm3 of cubic boxes volume. All simulation boxes have a 1.0 nm spacing distance around the system surfaces. Each system was then neutralized by adding Na+ counter ions to insulin and insulin-β-CD complex systems to maintain overall charge. After that, the energy minimization was performed using the steepest descent algorithm for 1000 steps to remove bad contacts. Following steepest descent energy minimization, the equilibrations of the system were then conducted in two phases: (1) NVT ensemble (constant number of particles, volume and temperature), a 100 ps position-restrained of insulin, and β-CD simulation at 300 K was carried out using a Berendsen thermostat to ensure the proper stabilization of the temperature; (2) NPT (constant number of particles, pressure, and temperature) ensemble, a 100 ps position-restrained of insulin and β-CD simulation at 300 K and 1 bar was carried out using a Parrinello–Rahman barostat pressure coupling to stabilize the system. Finally, a 90 ns MD simulation was carried out at 1 bar and 300 K. LINCS algorithm (Hess *et al*., 1997) were used to constrain the covalent bonds while the electrostatic interactions were treated using the Particle Mesh Ewalds (PME) method (Abraham and Gready, 2011). The cutoff radii for Coulomb and van der Waals interactions were set to 12 Å and were updated every 2 fs. Finally, the coordinates were recorded every 500 steps. The analyses of MD trajectories were done using g_rms, g_gyrate, g_rmsf, and g_hbond to obtain the root mean square deviation (RMSD), radius of gyration (Rg), root mean square fluctuation (RMSF), and the number of hydrogen bonds between insulin and β-CD. Grace-5.1.23 (http://plasma-gate.weizmann.ac.il/Grace/) program was used for the graph plotting along the simulation trajectories.

RESULTS AND DISCUSSION

Multiple molecular docking:

We performed the multiple molecular docking for 1:1, 1:2, 1:3 and 1:4 insulin to β-CD ratio. Table 1 shows the results of the docking summary on insulin for different insulin to β-cyclodextrin ratios. Docking calculation computed the binding energy which builds up the intermolecular energy (constituted by van der Waals energy, hydrogen bonding bond energy, desolvation energy and electrostatic energy) and internal energy (Chandler, 2005). Results show that the 1:3 insulin-β-CD complex has the lowest binding free energy (-1.67 kcal mol-1). The variation in the binding free energies occurs due to the differences of hydrophobic residue interactions and hydrogen bonding formation in each conformation. The conformation which was more stabilized by hydrophobic interactions tends to have lower binding energy, consequently showing the most stable conformation.

Table 1: Docking summary of energy of each insulin-β-CD ratio complex.

Insulin-β-CD ratio	Binding free energy (kcal/mol)	Hydrogen bonds	Hydrophobic interactions
1:1	-0.15	Asn24, His26, Val23, Gln25	Phe22, His30, Leu26
1:2	-1.01	Ser12, Tyr14 (2), Asn18 (2), Glu17 (3)	Leu13, Gln15
1:3	-1.67	Glu41 (2), Arg42	Asn21, Tyr36, Leu37, Gly40
1:4	-0.85	Asn24 (2), Phe22 (2)	Ile10, Ser12

The hydrogen bonds formations and hydrophobic interactions at each binding site in the complexations were shown in Fig. 2 and Fig. 3, respectively. The standard H-bonding criteria were set as diameter (H···A) of 3.0 Å and angle of (X−H···A) of □ 90°. As we can see in the figures, the hydrogen bonds formation in binding site I involved the bonds between the primary hydroxyl groups of β-CD and the amino acid residues Asn24, His26, Val2 and Gln25. When the docking process was further continued to the second docking cycle the second β-CD was able to form more hydrogen bonds with binding site II. The formation of hydrogen bonds between primary hydroxyl groups of β- CD was formed between Ser12, Tyr14 (2), Asn18 (2) and Glu17 (3). The higher formation of hydrogen bonds at binding site II was in line with the lower value of binding energy. The third β-CD was then docked and resulted in the lowest binding energy due to the formation of many hydrophobic residues in the complexation which was between Asn21, Tyr36, Leu37 and Gly40 and CD cavity. When we continue to dock the fourth β-CD the hydrogen bonds formation between primary hydroxyl groups in β-CD was formed between Asn24 (2) and Phe22 (2). However, this complex has form contacts only with Ile10 and Ser12 as hydrophobic interactions. The binding energy also increases to a much higher value

than 1:3 insulin-β-CD complexation. The attempt to dock the 5th β-CD could not locate any other possible binding site on insulin. As a result, we found that 1:3 insulin-β-CD complex system was the most stable conformation which was stabilized both by hydrogen bonds and hydrophobic interactions.

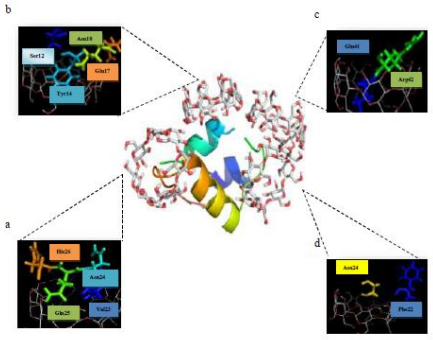

Fig. 2: The encapsulation of β-CD to insulin by hydrogen bonds formations at (a) binding site I (b) binding site II (c) binding site III and (d) binding site IV of insulin-β-CD complexes.

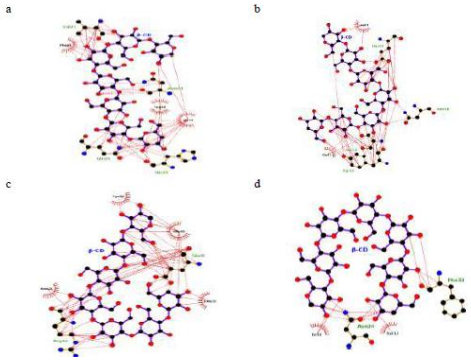

Fig. 3: Schematic representation of the hydrophobic interaction between insulin with β-CD at (a) binding site I (b) binding site II (c) binding site III and (d) binding site IV produced using the LigPlot program. Hydrophobic contacts are indicated by an arc with spokes radiating towards the ligand atoms they are in contact. The interacted atoms are spokes radiating back.

Molecular dynamics simulation:

MD simulations were performed for the insulin-β-CD complex as well as for the free insulin. This provided a better picture of the stability of insulin-β-CD complex on nanosecond time scale. The 1:3 insulin-β-CD complex was selected from the lowest binding energy which corresponded to the most stable conformation from results in the previous docking calculation. The complex model and the free insulin are subjected to 90 ns MD simulations in order to find out the stability of insulin in the presence of β-CDs. The results have been analyzed using the root mean square deviations (RMSDs), radius of gyrations (Rg), root mean square fluctuations (RMSFs) and hydrogen bond patterns.

Conformational stability:

The RMSD graph for both systems was shown in Fig. 4. After 80 ns, the insulin-β-CD complex system achieved a stable conformation throughout the simulation, with RMSD values of 0.65 ± 0.05 nm. Meanwhile, the free insulin system still not yet stable until the end of the simulation. The RMSD profile shows the increasing pattern for insulin-β-CD complex system until 40000 ps before it was finally stabilized. This is expected to occur due to the large conformational change of the residues in B chain insulin. Rg which is the average distance between an atom and its centre-of-mass at a given timestep, is also used to determine the number of atoms around during the simulation time. The average Rg value can be used to describe the size and compactness of the involved system (Dinner *et al*., 1999). Rg values for insulin-β-CD complex system was stabilized around 8000 ps, indicating that the equilibrium has been achieved. Initially the Rg values for both free and insulin-β-CD complex system was 1.0 nm, showing slight increases in the Rg value for both systems which was stabilized at 1.02 ± 0.01 nm. As shown in Fig. 5, the radius gyration of both systems different to each other, which clearly indicate that there are large conformational changes during the simulation.

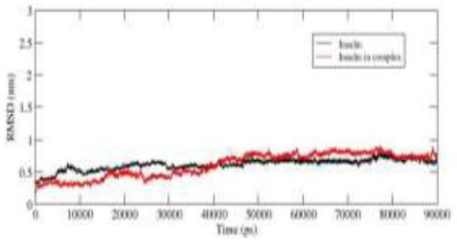

Fig. 4: Time dependence RMSD for free insulin system and insulin in complex system during 90 ns simulation.

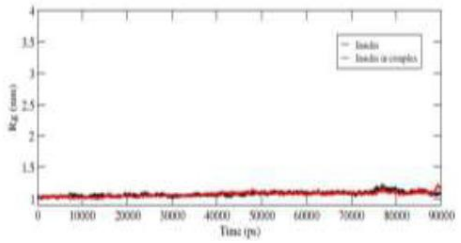

Fig. 5: Time evolution of the radius of gyration (Rg) during 90 ns of MD simulation of free insulin and insulin in complex.

Flexibility and dynamics behavior:

The RMSF of residues of insulin-β-CD in the trajectory is presented in Fig. 6. RMSF is used as a

mean to describe the flexibility differences among residues in a system. High RMSF value shows more flexibility whereas the low RMSF indicates limited movements during the simulation. In the present study, the residues in insulin which is consists of chain A (blue color) and chain B (red color) are numbered from one to fifty-one. The RMSF values of insulin residues of Ile2 (0.3008 nm), Asn21 (0.2999 nm) in chain A, Phe22 (0.4983 nm), Val23 (0.3677 nm), Asn24 (0.3379 nm), Lys50 (0.3691 nm), Ala51 (0.3651 nm) in chain B changed greatly which shows that the complex is most flexible at these positions. The result indicates the residues in chain B (red color) are involved with higher fluctuations. This is in keeping with previous reports of the structure activity studies of insulin which reported that the C-terminus of Chain B acts as the integral to receptor interaction (Pullen *et al.*, 1976). The previous report on molecular modeling of insulin analogues showed that the fluctuations may be due to the independent folding characteristic of insulin chains which occurs in the B chain of insulin (Berhanu and Masunov, 2012). Our results are also in agreement with the previously obtained NMR experimental data which explained that insulin exhibited a novel partial folding under amyloidogenic conditions (Hua and Wess, 2004). In addition, residues Asn21, Phe22, Val23 and Asn24 which exhibited higher fluctuation distances in RMSF profiles are also involved in the hydrogen bonding and hydrophobic interactions in docking calculations.

The analysis of the hydrogen bonds formation of insulin in 1:3 insulin-β-CD complex versus the simulation time is shown in Fig. 7. Overall, the highest numbers of intermolecular hydrogen bonds formations between insulin and β-CD is 10. On average, the total formations of hydrogen bonds formed are between 4 to 6 bonds. While the hydrogen bond interactions help in the stabilization of the complex, the hydrophobic interactions are still the main contributors which stabilized the complex.

The hydrophobic side chains of the insulin amino acids penetrate into the cyclodextrin cavity and lead to the formation of noncovalent inclusion complexes (Yoshida *et al.*, 1989) and enhance the stability of insulin-β-CD complex.

The 3D representation of the insulin-β-CD along the simulation time is shown in Fig. 8. All three β-CDs molecules stayed at the initial dock binding site of insulin until the end of the simulation period which confirms the reliability of the docking results in this work. A slight change in the movement may happen due to the flexiblility of the residues in insulin itself.

Conclusion:

The best ratio of insulin to β-CD were determined using the multiple molecular docking approach and our results show that up to three β-CDs can be bound to insulin. The molecular dynamic simulation results are in accordance with the docking results, which show the stability of the complex system when compared to the free insulin system. Both hydrogen bonds and hydrophobic interactions contribute to the complex stability. The flexibility of residues in insulin-β-CD complex is responsible to the structural change in insulin but the binding sites with β-CDs remains throughout the simulation. This flexibility is in keeping with the change in the radius of gyration, which shows the conformational changes of the insulin structure during the simulation period. The hydrogen bonds analysis along the trajectories indicates the contribution of hydrogen bonding formation in the stabilization of complex systems. Overall, the results of this study suggested that the complexation of insulin with β-CD is thermodynamically stable and provide a theoretical explanation to the new oral insulin delivery formulation.

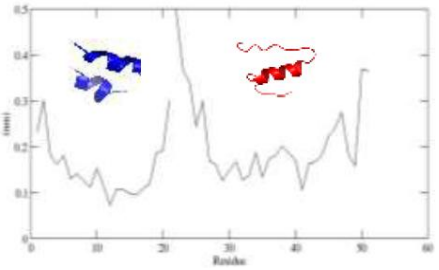

Fig. 6: Root mean square fluctuations (RMSFs) for the last 10 ns with respect to the initial structure of the free insulin and insulin-β-CD complex.

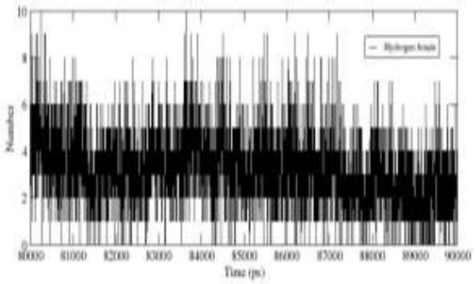

Fig. 7: Hydrogen bonds formation analysis between insulin and β-CD in 1:3 insulin-β-CD complex.

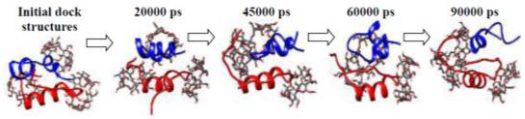

Fig. 8: A 3D representation of the 1:3 insulin-β-CD complex along the simulation. Insulin in ribbon (blue: A chain and red: B chain) while β-CD in stick.

ACKNOWLEDGMENT

The study was financially supported by the funding from Universiti Sains Malaysia, Penang through the Research University Grant (Grant no. 1001/PKIMIA/815099). The authors also gratefully acknowledged the technical assistance from Department of Chemistry, Faculty of Science, Universiti Putra Malaysia.and sanitization techniques.

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