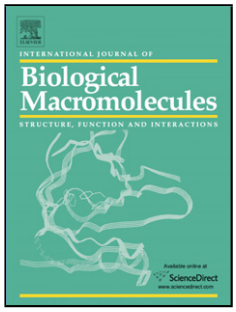

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Author: Hossein Soleymani Ali A. Saboury Ali A. Moosavi-Movahedi Fatemeh Rahmani Javad Maleki Saeid Yousefinejad Parvaneh Maghami

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Vitamin E induces regular structure and stability of human insulin, more intense than vitamin D_3

Hossein Soleymani[a], Ali A. Saboury[a,b,*], Ali A. Moosavi-Movahedi[a,b], Fatemeh Rahmani[c], Javad Maleki[d], Saeid Yousefinejad[e], Parvaneh Maghami[a]

[a.] Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran.

[b]. Center of Excellence in Biothermodynamics, University of Tehran, Tehran, Iran.

[c.] Department of Plant Science, Faculty of Life Science, Tarbiat Modares University, Tehran, Iran.

[d]. Department of Biology, Faculty of Basic Science, Hakim Sabzevari University, Sabzevar, Iran.

[e]. Department of Chemistry, Faculty of Basic Science, Shiraz University, Shiraz, Iran.

E-mail address other authors:

1- Hossein Soleymani: h_soleymani@ut.ac.ir

2- Ali Akbar Moosavi Movahedi: moosavi@ibb.ut.ac.ir

3- Fatemeh Rahmani: fatemehr_67@yahoo.com

4- Javad Maleki: javadmaleki394@yahoo.com

5- Saeid Yousefinejad: yousefinejad@ibb.ut.ac.ir

6- Parvaneh Maghami: p.maghami@ibb.ut.ac.ir

[*] Corresponding Author. Tel.: +98-21 66956984; fax: +98-21 66404680

E-mail address: saboury@ut.ac.ir (Ali Akbar Saboury).

Abstract

Changes in human environment and lifestyle over the last century have caused a dramatic increase in the occurrence of diabetes. Research of past decades illustrated that vitamin D and E have a key role in the improvement of diabetes by reducing oxidative stress, protein glycosylation, insulin resistance and also improving beta cell function. Binding properties and conformational changes of human insulin upon interaction with vitamins D_3 and E (α-tocopherol) were investigated by spectroscopy, differential scanning calorimetry (DSC) and molecular dynamic simulation. Tyrosine fluorescence quenching studies indicates changes in the human insulin conformation in the presence of vitamins. Binding constants of vitamins D_3 and E for human insulin were determined to be 2.7 and 1.5 ($\times 10^{-5}$ M^{-1}) and the corresponding average numbers of binding sites were determined to be 1.3 and 1.2, respectively. Far- and near-UV circular dichroism studies showed that vitamin E can significantly change the secondary and tertiary structures of human insulin *via* an increase in the content of α-helix structure. Results of DSC showed that both vitamins D_3 and E stabilize the structure of human insulin. Molecular dynamic simulation results indicated that vitamin D_3 decreases the helical and strand structural contents of human insulin, but vitamin E stabilizes more regular secondary structures such as helical and strand structural contents as shown by experimental results.

Keywords: Human insulin, Vitamin D_3, Vitamin E, Differential scanning calorimetry, Molecular dynamic simulation, Circular Dichroism

1. Introduction:

The human environment and lifestyle have been changed over the last century, which lead to a dramatic increase in the occurrence of diabetes mellitus [1]. The past decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide [2, 3]. Diabetes is the accumulation of a number of metabolic diseases specified by hyperglycemia resulting from faults in insulin secretion, insulin action, or both [4]. Research of past decades illustrated that insulin has a central role in the advancement of pharmacology, peptide chemistry, cell signaling and structural biology [5]. Insulin is a peptide hormone that exists in the blood and it regulates the metabolism of carbohydrates by promoting of glucose absorption from blood to skeletal and fat tissues. Human insulin is a peptide containing 51 amino acids in two polypeptide chains linked by three disulfide bonds [6]. Only at low concentration (<0.1 µM, ~0.6 µg/ml) insulin exists as a monomer. Dimerization occur at higher concentrations and in the presence of zinc ions, in pH range 4-8, three dimers assemble into a hexamer at concentrations >0.01 mM [7].

Many confirmed or experimental remedies for type 1 and type 2 diabetes (e.g., immunomodulation [8, 9], pancreas or islet transplants [10, 11], bariatric/metabolic surgery [12-14]) and improving insulin stability through amino acid substitution [15]are of curative purpose or have been illustrated in the media as a possible treatment. However, describing cure or remission of diabetes is not as straightforward as it may seem [16].

In the last decade, vitamin D has scintillated wide interest in the prevention and pathogenesis of diabetes. As the main regulator of calcium homeostasis, vitamin D directly and/or indirectly improves glucose tolerance, exocytosis of insulin *via* activating calcium-dependent endopeptidases and it also improves glucose secretion [17, 18]. Vitamin D could also prevent type 2 diabetes through its role as an effective antioxidant [19, 20]. Recently, it has been shown that the non-insulin dependent diabetes mellitus patients are generally

associated with vitamin D_3 deficiency, which is significantly related to the oxidative stress conditions in this group of patients [21]. Recent investigation by Roya Kelishadi et al has demonstrated that vitamin D supplementation is inversely associated with insulin resistance and also some cardiometabolic risk factors in children, which it may have beneficial consequences on controlling some complications of childhood obesity [22]. Independently association of the lowest concentration of 25(OH)D with all-cause mortality in the general population is also known [23]. In 2015, Truong-Minh Pham et al suggested that improvements in vitamin D status diminish the risk for insulin resistance and hence may contribute to the primary prevention of non-insulin dependent diabetes mellitus patients and cardiovascular disease [24]. Also, vitamin D supplementation could reduce insulin resistance in non-insulin dependent diabetes mellitus patients with significant improvements in serum fasting plasma glucose (FPG) and insulin [25].

On the other hand, daily oral vitamin E supplements reduce oxidative stress and protein glycosylation and finally improve insulin action by changes in the plasma oxidized glutathione-glutathione (GSSH-GSH) ratio that occur after its oral administration in non-insulin dependent diabetes mellitus patients [26]. An improvement of glucose control and insulin sensitivity was found in Type 2 diabetic patients remedied by high doses of vitamin E [26]. The findings show that people with a low serum α-tocopherol concentration had an elevated subsequent risk of insulin dependent diabetes mellitus [27]. Recently, it has been shown that the lower dietary vitamin E intake during the second trimester is correlated with insulin resistance and hyperglycemia in pregnancy among women using daily multivitamin supplementations [28]. Also, it is known that vitamin E tocotrienols ameliorate insulin sensitivity through activating peroxisome proliferator-activated receptors in both insulin dependent diabetes mellitus and non-insulin dependent diabetes mellitus patients [29]. It is

discovered that combined vitamin E and insulin treatment additively prohibit the progression and development of renal damage in diabetic rats [30].

With all this said, a question arises whether how these vitamins in serum interact with insulin and how change structure and stability of insulin. It is known that vitamin D and E have a positive effect on diabetes, but the interaction of these vitamins and insulin in the blood is still unclear. Vitamin D and E are fat-soluble vitamins that exist in the blood in the active form of vitamin D_3 and α-tocopherol. In this paper, for the first time, we investigated the interactions between vitamin D_3 and α-tocopherol with human insulin individually to examine change in the insulin structure and stability.

2. Materials and Methods

2.1 Materials

Recombinant human insulin (91077C) and vitamin E (α-tocopherol, T3251) were obtained from Sigma-Aldrich. Potassium hydrogen phosphate (137010) and ethanol (986) were purchased from Merck. Vitamin D_3 (cholecalciferol) from Supleco analytical company was used. Deionized water was filtered using a Millipore system (Millipore, Billerica, MA) and used for all samples. All human insulin samples were prepared in 7 mM phosphate buffer adjusted to pH 7.4 with sodium hydroxide and/or perchloric acid. All samples were made from fresh stock solutions. The final concentration was determined by an extinction coefficient of 6200 $M^{-1}cm^{-1}$ at 276 nm for human insulin [31], 18300 $M^{-1}cm^{-1}$ at 265 nm for vitamin D_3 [32] and 3270 $M^{-1}cm^{-1}$ at 292 nm for vitamin E [33].

2.2 Methods

2.2.1 Fluorescence spectroscopy

Tyrosine intrinsic fluorescence quenching of insulin was measured in the absence and in the presence of different concentrations of vitamin D_3 and α-tocopherol individually at 25°C

(room temperature) by Carry Eclipse (Varian, Australia) Spectrofluorimeter on the excitation wavelength of 276 nm. To ensure, all experiments were repeated three times and the reported data are averages of the results. Insulin with concentration of 15 µM was titrated with small aliquots of vitamin D_3 stock solution (54800 µM) and insulin with concentration of 25 µM was titrated with vitamin E (α-tocopherol) stock solution (3000 µM) in ethanol. At the end of the titration the molar ratio of vitamin D_3/ insulin reached 8.67 and molar ratio of vitamin E/ insulin reached 2.68 and the concentration of ethanol was not more than 3% v/v. The mixture of insulin with vitamin incubated for 4 minutes to form insulin and vitamin complex before measurement of the fluorescence intensity. Since vitamin D_3 and α-tocopherol have absorption in the study range, absorption of UV light by the same volumes of added vitamins to human insulin was measured by Carry 100 Bio Varian UV-Vis spectrophotometer in the buffer and the fluorescence intensity was corrected according to the following equation [34] [equation 1]:

$$= \times 10^{(\frac{+}{2})} \qquad [1]$$

Where F and F_{init} are corrected and initial fluorescence intensity, respectively, A_{ex} is the absorption of vitamins in the excitation wavelength and A_{em} is the absorption of vitamins in the emission wavelength.

To extract the detailed spectral and concentration information of pure species from the overlapping fluorescence spectra for interaction of α-tocopherol with human insulin, the chemometric analysis (the multivariate curve resolution-alternating least squares method (MCR-ALS)) was done [35].

2.2.2 Circular dichroism (CD)

Vitamin induced changes in the secondary and tertiary structure of insulin at room temperature (25^{o}C) were followed from 260 to 190 nm (far UV-CD) and 320 to 260 nm (near UV-CD) by an Aviv 215 Spectropolarimeter (Lakewood, New Jersey, USA). Sample

preparation was performed with molar ratio of 4.35 and 8.7 for vitamin D_3 and molar ratio of 1.35 and 2.7 for α-tocopherol in respect to insulin. The filtered insulin-vitamin samples were scanned in a 0.1 cm cuvette using a bandwidth of 2.0 nm, a response time of 2 s, a data pitch of 0.5 nm. The obtained data were converted to molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), by CDNN software analysis [36].

2.2.3 Differntial scanning calorimeter (DSC)

The DSC measurement was done using differential scanning calorimeter (N-DSC II, Calorimetry sciences, Utah USA). All protein and protein-vitamin complex scans were performed with 7 mM phosphate buffer in the reference cell from 25 to 95 ^oC at a heating rate of 1 ^oC/min and an excess pressure of 0.2 Mpa. First, a solution of insulin with concentration of 345 µM (2 mg/ml) was placed in the sample cell. Then, a mixture of 345 µM insulin with intermediate level and the saturation level of vitamin/protein molar ratio (vitamin D_3 at the molar ratio of 4.35, 8.7 and α-tocopherol at the molar ratio of 1.35, 2.7 vitamin/protein) was used to form the complex (similar to CD spectroscopy method). To concentration normalization, a buffer-buffer reference was subtracted from each sample scan prior. Thermodynamic parameters were calculated from thermograms using CpCale software (version 2.1) supplied by the manufacturer. Reversibility was checked by cooling the heated samples and repeating the experiment.

2.2.4 Dynamic Light Scattering (DLS)

The size of the complexes in the added time and 24 hours later were measured by the Zeta plus (Brookhaven Instruments, USA). Samples prepared by the same method of CD and DSC.

2.2.5 Molecular Dynamic Simulation

Molecular docking was done using AutoDock Vina with standard parameters [37]. Crystal structure of human insulin was obtained from the Brookhaven Protein Data Bank (PDB code 3INC) and ligand structures were obtained from the Brookhaven Protein Data Bank (PDB

code 3C6G for vitamin D_3 and 3CXI for α-tocopherol) and optimized by VMD program[38]. The MD simulations were performed using the Gromacs 4.5.5 package and energy was calculated by Gromos96 53a6 force field. The ligands topologies were generated by the PRODRG server and reparation. Insulin-VD3 and insulin-VE solvated with SPC water which left 1.0 nm space around the solute. All-atom MD simulations were also performed. Some counter ions were added to system to neutralize. Steepest descent energy minimization was carried out down to a maximum gradient of 1000 kJ $mol^{-1}nm^{-1}$. The system was simulated in two steps, 100 ps of position restrained dynamics using LINCS algorithm followed by full 10 ns standard MD. The system was coupled to a Berenson's thermostat method with the reference temperature at 300 K. All the simulations were carried out at standard pressure of 1.0 bar with a coupling constant of 300 ps for pressure. It should be noted that during this time, positions of all bonds were constrained. PME electrostatics were applied using the Lennard–Jones cutoff of 1.4 nm and a coulomb cutoff of 0.9 nm. MD simulation was done during 10ns for all minimized structures for each of two simulations with time step of 2 fs. On all the trajectories, RMSD was always calculated. Images were created using Python Molecular Viewer (PMV) and the program LigPlot+ v.1.0, which generates schematic 2-D representations of protein ligand complexes from the PDB file input [39, 40].

3. Result and Discussion

3.1 Fluorescence spectroscopy

The binding of a ligand to a protein may instantly affect the fluorescence of tryptophan and/or tyrosine residues by physically interacting with the fluorophore or by acting as a quencher, thereby changing its accessibility to solvent and/or the polarity of its environment. Both direct and indirect effects may result either in the quenching or increment of fluorescence intensity and/or blue or red spectrum shift [41]. Human insulin has four Tyr residues at positions 14, 19 in chain A and also 16, 26 in chain B, but it hasn't any Trp residue; so the maximum

absorbance of human insulin occurs at 276 nm. A typical spectrofluorimetric titration of human insulin with vitamin D_3 and α-tocopherol with excitation held at 276 nm is shown in Fig. 1 (a and b). The decrease in the fluorescence intensity is due to the conformational change and exposure of hydrophobic part of the human insulin such as tyrosine residue to the solvent. The fluorescence spectrum of the native human insulin shows a maximum at 303 nm in phosphate buffer (7mM and pH 7.4). Fig. 1a shows the fluorescence spectrums of human insulin (15 µM) quenched by vitamin D_3 (0 to 121.5 µM) and Fig. 1b shows the fluorescence spectrums of human insulin (25 µM) quenched by α-tocopherol (0 to 67 µM). As shown in Fig. 1, a higher vitamin concentration leads to a greater reduction in the intrinsic fluorescence emission of human insulin, finally quenching it. Maximal quenching was reached at 8.64-fold molar excess of vitamin D_3 and 2.67-fold molar excess of α-tocopherol for human insulin. After the addition of α-tocopherol to human insulin, the fluorescence band of insulin at 303 nm decreased, broadened and red-shifted, implying the formation of a complex between α-tocopherol and human insulin (Fig. 1b). Simultaneously, the fluorescence band at 324 nm intensified, and an isobestic point at 318 nm in the fluorescence spectra can be clearly observed. In fluorescence spectroscopy, isosbestic point is a specific wavelength at which the total intensity of a sample does not change during a chemical reaction or a physical change of the sample. The appearance of isosbestic point in the absorption/emission spectrum typically indicates that the two species involved are related linearly by stoichiometry and vary in concentration contribute to the absorption/emission around the isosbestic point. As shown in Fig. 1 (c and d) increase of insulin-VE complex concentration occur simultaneously with decrease in human insulin concentration during the alpha-tocopherol titration.

(Fig. 1)

The Chemometrics results demonstrated that by increasing the α-tocopherol concentration, a chemical intermediate is created. This intermediate species converted to a new product,

named ″insulin-VE complex″ in the saturation concentration of α-tocopherol. It can be realized that insulin-VE complex may have different structural features compared with insulin. Also, the experimental fluorescence peak of human insulin is matched very accurately to deconvoluted peak of the first species that represents the goodness of deconvolution. Figures 1c and d shows Chemometrics results.

Quenching type can be distributed into two broad categories of static quenching and dynamic quenching, according to whether the relationship between concentration of the quencher [Q] and intensity of fluorescence emission (F) completely fit the Stern-Volmer equation (dynamic) or Perrin equation (static). In order to discern the fluorescence quenching mechanism and quenching parameters, the fluorescence quenching data of human insulin was analyzed using the Stern-Volmer and modified Stern-Volmer equations. From these results, it can be concluded that vitamins bound to human insulin and the insulin-VD_3 and insulin-VE complex formed, resulted in a fluorescence quenching of the fluorophore. This result indicates that the probable quenching mechanism of insulin-vitamins binding reactions are initiated by compound formation rather by dynamic collision.

The values of f_a for insulin-VD_3 and insulin-VE were found to be 1.35 and 1.52 indicating that 74% and 66% of the total fluorescence of human insulin were accessible to quencher and the others 26% and 34% (for human insulin, respectively) are not affected by vitamin D_3 and α-tocopherol (see supplementary appendix). The Stern-Volmer quenching constant were calculated and listed in Table 1. According to obtained results, n is 1.3 ± 0.03 and K_b is equal to (2.7 ± 0.12) × 10^5 M^{-1} for insulin-VD_3 complex and n is 1.2 ± 0.03 and K_b is equal to (1.5 ± 0.14) × 10^5 M^{-1} for the insulin-VE complex. ΔG^o was determined according to $\Delta G^o = -RT \ln K_b$ formula at 25°C, that is equal to -7.4 kcal mol^{-1} for insulin-VD_3 complex and that is equal to -7.04 kcal mol^{-1} for the insulin-VE complex.

(Table 1)

3.2 Circular Dichroism (CD)

The CD is an ideal technique for monitoring conformational changes in proteins, which can arise as a result of changes in experimental parameters such as temperature, ligand binding, pH, among others [42-45]. To study the effects of interaction with vitamin D_3 and α-tocopherol on human insulin structure, we used far-UV CD. The far-UV CD and near-UV CD spectrum of human insulin in the absence and presence of different concentration ratio of vitamin D_3 (0, 4.35 and 8.7 μM) and α-tocopherol (0, 1.35 and 2.7 μM) are shown in Fig. 2 (a and b). The human insulin CD spectrum is typical of a protein that is composed of α-helix structure and shows two minimum at 208 nm ($[\theta_{208}]$) and 222 nm ($[\theta_{222}]$) [44, 46]. Figure 2a shows that interaction of vitamin D_3 with insulin led to a slight decrease in the size of the negative CD signal within 200-230 nm, reflecting the decreased compactness and stability of the helical regions of the protein. But the interaction of α-tocopherol with insulin led to an intense increase in the size of the negative CD signal within 200-230 nm, reflecting the increased compactness and stability of the helical regions of the insulin as it is known that phenol stabilizes more α-helix in insulin structure [47].

(Table 2)

The near-UV CD spectra of proteins occur from the environment of each aromatic amino acid side chain further possible contributions from disulfide bonds, or non-protein cofactors that may absorb in this spectral region, afterwards giving information about the tertiary structure of the protein [48]. The near-UV CD spectrum of human insulin alone shows a pattern of negative peak between 260 and 290 nm (Fig. 2B) due to the presence of aromatic residue: four tyrosines (Tyr 14a, 19a, 16b and 26b) and three phenylalanines (Phe 1b, 24b and 25b). Trp signals generally occur between 250 and 300 nm, whereas Phe and Tyr typically do not absorb above 270 nm and 290 nm, respectively [44, 49]. Therefore, the negative bands at 285 nm and 293 nm can be assigned to asymmetrically perturbed tryptophans while peaks

below 280 nm are likely the consequence of the chiral environment of Tyr and Phe residues [49]. According to a near-UV CD results, the tertiary structure of insulin upon the interaction of vitamin D_3 do not change, but the tertiary structure of insulin upon the interaction of α-tocopherol change and α-tocopherol led to a significant increase in the size of the negative CD signal over the range of 260-300 nm, indicating that the rigidity of insulin is increased. Consequently, the insulin-VE complex becomes more stable, which is in agreement with far-UV CD data.

(Fig. 2)

3.3 Dynamic light scattering (DLS)

DLS results indicate that the diameter of human insulin and its complex with vitamin D_3 and α-tocopherol were 5.4 nm, 5.9 nm and 5.0 nm, respectively. After 24 hours of complex formation, the diameter of insulin-VD_3 and insulin-VE were 119 nm and 107 nm, respectively and it is much larger than the protein. A nanoparticle of homogenized whey protein and vitamin D_3 with similar size (110 nm) and complex of α-lactalbumin with vitamin D_3 with similar size (125 nm) were reported recently [50, 51]. This change might be the result of encapsulation of α-tocopherol and vitamin D_3 in human insulin and also of protein-protein interactions. When vitamins bind with insulin, hydrophobic patches get exposed, enabling hydrophobic interactions between insulin molecules leading to larger insulin complexes [50, 52]. Decreasing of insulin dimension from 5.4 nm to 5.0 nm upon interaction with α-tocopherol shows that protein compactness and rigidity are increased as CD had shown.

3.4 Differential scanning calorimetry (DSC)

Stability of the human insulin and its complex with vitamin D_3 and α-tocopherol was studied by DSC technique. DSC thermogram shows the excess heat capacity as a function of temperature (Fig. 6). Parameters obtained by DSC including enthalpy change of denaturation (ΔH^o), entropy change of denaturation (ΔS^o), transition temperature (T_m) and heat capacity

change upon unfolding (ΔC_p) are presented in Table 2. A comparison of the thermograms shows that the T_m of human insulin decreases in the presence of vitamin D_3 and it increases at the presence of intermediate molar ratio of α-tocopherol.

Analysis of DSC thermograms reveals that the T_m and ΔG° of denaturation of human insulin are 73.1 °C and 8.89 kcal.mol^{-1}, respectively. In the presence of 1.35 (intermediate) fold of α-tocopherol in respect to human insulin, those parameters reached to 74.7 °C and 7.49 kcal.mol^{-1}. In the presence of 2.7 (saturation) fold of α-tocopherol in respect to human insulin, those parameters decreased to 72.7 °C and 6.80 kcal.mol^{-1}, respectively. Also, in the presence of 4.35 (intermediate) and 8.70 (saturation) fold of vitamin D_3 in respect to human insulin, those parameters decreased to 72.2 °C, 6.64 kcal.mol^{-1} and 70.1 °C, 3.85 kcal.mol^{-1}, respectively. Decrease in the T_m can be related to vitamin D_3 binding to human insulin or the increase of alcohol concentration in solvent and change in solvent conditions. An experiment was done in the absence of vitamins but the presence of so much alcohol as it was present at the last stage of the previous experiment, to examine the effect of ethanol. As shown in Fig. 3, related to ethanol, this concentration of ethanol strongly decreases the T_m and ΔG° of denaturation of insulin (66.3 °C and 1.98 kcal.mol^{-1}). Therefore, in the presence of the vitamin D_3, ethanol has contributed in complex destabilization. It seems that ethanol has a destroyer effect on the structure of human insulin and lead insulin to fibrillation [53, 54], while vitamin D_3 and α-tocopherol stabilize structure of human insulin more against ethanol. Moreover, current experiment about the effect of ethanol in T_m, shows that the α-tocopherol increases the stability of insulin, although thermogram does not show. The change in heat capacity between the unfolded and native states (ΔC_p) can be related to the exposure or concealment of hydrophobic surface areas. ΔC_p of human insulin decreases in the presence of both vitamins (Table 2). Decreases of change in heat capacity shows more concealment and covering of hydrophobic parts for proteins in complex [55]. On the other hand, denaturation enthalpy

(ΔH^o) of all complexes decreased when compared to free protein. It seems that enthalpy changes because of intermolecular interactions affected to resulting enthalpy. It can be said that at different concentrations of vitamin D_3 and α-tocopherol, interaction of vitamins with human insulin makes the hydrophobic parts of the protein more restricted what is causing decreases in protein-protein hydrophobic interactions between insulin molecules.

(Fig. 3)

The difference in Gibbs energy (ΔG^o) of denaturation is calculated from the modified Gibbs-Helmotz equation [56, 57] [equation 2]:

$$\Delta \quad = \Delta H \quad (1 - —) + \Delta \quad [(\quad - \quad) - \quad \ln (—)] \qquad [2]$$

Where ΔH^o_m is the value of ΔH^o at T_m ($\Delta H^o(T_m)$). DSC results indicate that ΔG^o of denaturation of human insulin (8.89 kcal mol^{-1}) strongly decreased in the presence of ethanol alone (1.98 kcal mol^{-1}), but in the presence of saturation level of vitamin D_3 and α-tocopherol, ΔG^o of denaturation increased to 3.85 kcal mol^{-1} and 6.83 kcal mol^{-1}, respectively. It seems that decrement in ΔG^o of denaturation of human insulin is influenced ethanol more than both vitamins. Finally, reversibility test shows that in the presence of vitamin D_3, human insulin is less reversible than native protein, but α-tocopherol almost did not change reversibility of insulin (Table 2).

3.5 Molecular dynamic simulations

During the docking and molecular dynamic simulation, the dimeric insulin structure was used to study interaction with vitamin D_3 and α-tocopherol [58, 59]. Blind docking revealed that human insulin has two binding regions for vitamin D_3 and α-tocopherol, but local docking in both of the regions shows that one has a better score of binding. According to AutoDock calculation, the binding energy of vitamin D_3 and α-tocopherol were -6.9 and -6.5 kcal mol^{-1}, respectively that are in a good agreement with fluorescence results. Pursuant to Figure 4, Van der Waals and hydrophobic interactions play the major role in the binding of vitamin D_3 and

α-tocopherol to human insulin. Based on the results of the modeling, dominant interaction is hydrophobic (figures 4a and b). By analyzing the binding energy, certain insulin-vitamin complexes were selected for simulation studies.

(Fig. 4)

RMSD results during molecular dynamic simulation illustrated that the absolute structure of insulin-VD_3 complex and insulin-VE complex were stable during running time and RMSD in the insulin-VE complex is larger than insulin-VD_3 complex where is consistent with the intense conformational change of complex. An average RMSD value for insulin-VD_3 and insulin-VE complexes obtained equal to 2.0 nm and 3.7 nm, respectively (Fig. 5). This result indicated that the conformation of protein-vitamin complexes have remained stable during simulation. Although, RMSD of insulin-VE complex looks very high, which it related to strong secondary and tertiary structure change of protein, as it is shown by far and near CD results.

(Fig. 5)

Molecular dynamic simulation results indicated that vitamin D_3 decreases helix and strand structure of human insulin and increases random coil, but α-tocopherol stabilizes more regular secondary structures such as helical and strand structures as experimental results was shown. According to the literature, phenols are causing increasing of helical structure in insulin [47, 60, 61]. Figure 6 (a and b) shows the change in secondary structure of human insulin calculated by the STRIDE online server [62] and UCSF Chimera tools [63]. It seems changing in human insulin structure causes decrease of vitamin D_3 bend, but α-tocopherol in the insulin-VE complex is turned and bent itself. Figure 7 shows the vitamin binding point before and after MD for insulin-D_3 (a and c) and insulin-VE (b and d) complex. In Figure 7, bond distances >4 Å are related to Van der Waals and hydrophobic interactions.

(Fig. 6 and 7)

4. Conclusion

This study shows that vitamin D_3 and α-tocopherol can be bound to human insulin and quench the fluorescence spectra of the protein. The binding constants of vitamin D_3 and α-tocopherol with human insulin were calculated 2.7 and 1.5 ($\times 10^{-5}$ M^{-1}) and the corresponding average numbers of binding sites were 1.3 and 1.2, respectively. The Chemometrics results demonstrated that by increasing the α-tocopherol concentration, a chemical intermediate is created and in the saturation concentration of α-tocopherol, this intermediate species converted to the new product is named "the insulin-VE complex". Far- and near-UV CD studies showed that α-tocopherol induces conformational changes in the secondary and tertiary structure of human insulin and stabilizes it by an intense increasing the content of α-helix in the secondary structure of protein, but vitamin D_3 decreases the content of α-helix in the secondary structure of human insulin. Binding of vitamin D_3 and α-tocopherol yielded complex particles with a size of 5.9 and 5.0 nm that is approximately equal to the insulin dimension (5.4 nm). The docking results indicated that insulin has one binding site for vitamin D_3 and α-tocopherol and the binding energy of vitamin D_3 and α-tocopherol obtained -6.9 and -6.5 kcal mol^{-1}, respectively that are in a good agreement with fluorescence results. The absolute structure of insulin-VD_3 complex and insulin-VE complex were stable during running time. Molecular dynamic simulation results indicated that vitamin D_3 decreases helix and strand structure of human insulin and increases random coil, but α-tocopherol stabilizes more regular secondary structures such as helical and strand structures as experimental results was shown. Results of DSC thermograms show that the T_m of human insulin decreases in the presence of vitamin D_3 and it increases in the presence of the intermediate molar ratio of α-tocopherol. It seems that ethanol has a destroyer effect on the structure of human insulin and lead insulin to fibrillation [53, 54], while vitamin D_3 and α-tocopherol stabilize structure of human insulin more against ethanol. It concluded that vitamin E and D bound to human

insulin and stabilize its structure, so may can improve insulin half-life in blood flow and control diabetes disease with the consumption of these natural vitamins.

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7. Captions

Fig. 1. (a) Fluorescence spectra of human insulin in the absence and presence of different concentrations of vitamin D_3 (0 to 121 µM) in 7 mM phosphate buffer at pH 7.4 at 25 C. **(b)** Fluorescence spectra of human insulin in the absence and presence of different concentrations of α-tocopherol (0 to 201 µM) in 7 mM phosphate buffer at pH 7.4 at 25 C. **(c)** Fluorescence spectra deconvolution of human insulin with α-tocopherol. **(d)** Creation and conversion of biological intermediate from human insulin to insulin-VE complex extracted from fluorescence spectra deconvolution.

Fig. 2. (A) Far-UV CD spectra of 34.5 µM human insulin measured in the absence (a) and presence of different concentrations of vitamin D_3 [150 µM (b) and 300 µM (c)] and α-tocopherol [46.5 µM (d) and 93 µM (e)] in 7 mM phosphate buffer at pH 7.4 at 25 ^{o}C. **(B)** Near-UV CD spectra of 87 µM human insulin measured in the absence (a) and presence of different concentrations of vitamin D_3 [378.5 µM (b) and 757 µM (c)] and α-tocopherol [117.5 µM (d) and 235 µM (e)] in 7 mM phosphate buffer at pH 7.4 at 25 ^{o}C.

Fig. 3. DSC thermograms of 345 µM human insulin in phosphate buffer 7 mM, pH 7.4 (Insulin) and at the molar ratios of 4.35 (Insulin-VD_3-1), 8.7 (Insulin-VD_3-2) for vitamin D_3 and at the molar ratios of 1.35 (Insulin-VE-1), 2.7 (Insulin-VE-2) for α-tocopherol.

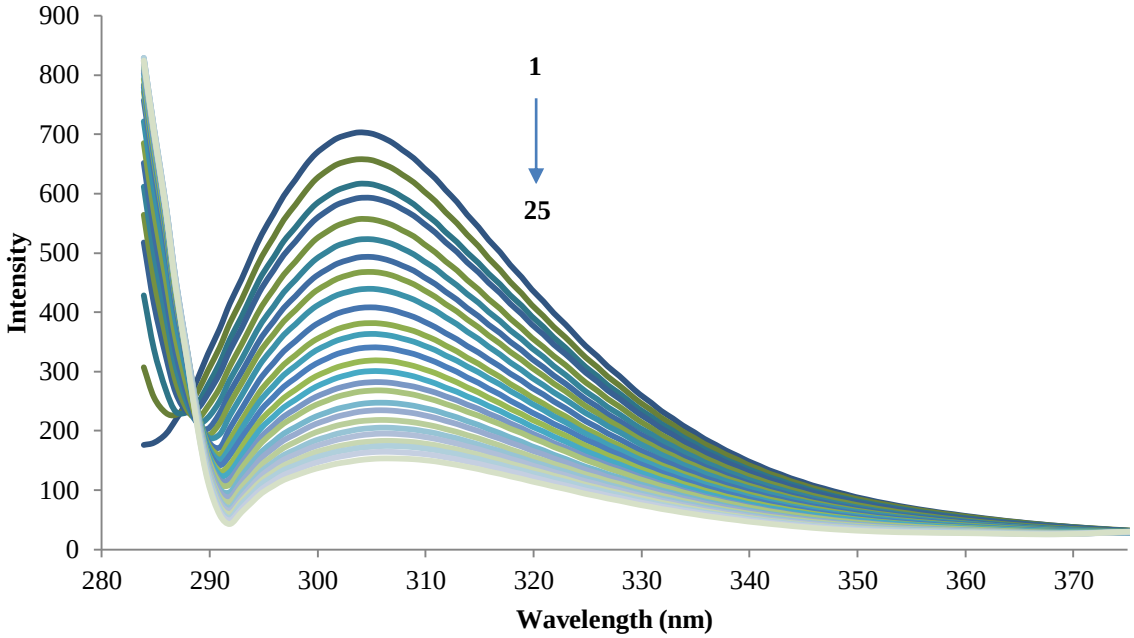
Fig. 4. LigPlot+ diagram of the interaction between human insulin and vitamin D_3 **(a)** and α-tocopherol **(b)**. Hydrophobic interaction plays the major role in the binding.

Fig. 5. The RMSD of backbone atoms of human insulin in complex with vitamin D_3 and α-tocopherol during 1000 picoseconds simulation.

Fig. 6. (a) Secondary structure of human insulin in complex with vitamin D_3 before and after MD calculated by the STRIDE online server (right) and UCSF Chimera tools (left). **(b)** The secondary structure of human insulin in complex with α-tocopherol before and after MD calculated by the STRIDE online server (right) and UCSF Chimera tools (left).

Fig. 7. (a) Binding point of vitamin D_3 to human insulin before MD. **(b)** Binding point of α-tocopherol to human insulin before MD. **(c)** Binding point of vitamin D_3 to human insulin after MD. **(d)** Binding point of α-tocopherol to human insulin after MD.

Fig. 1. (a)



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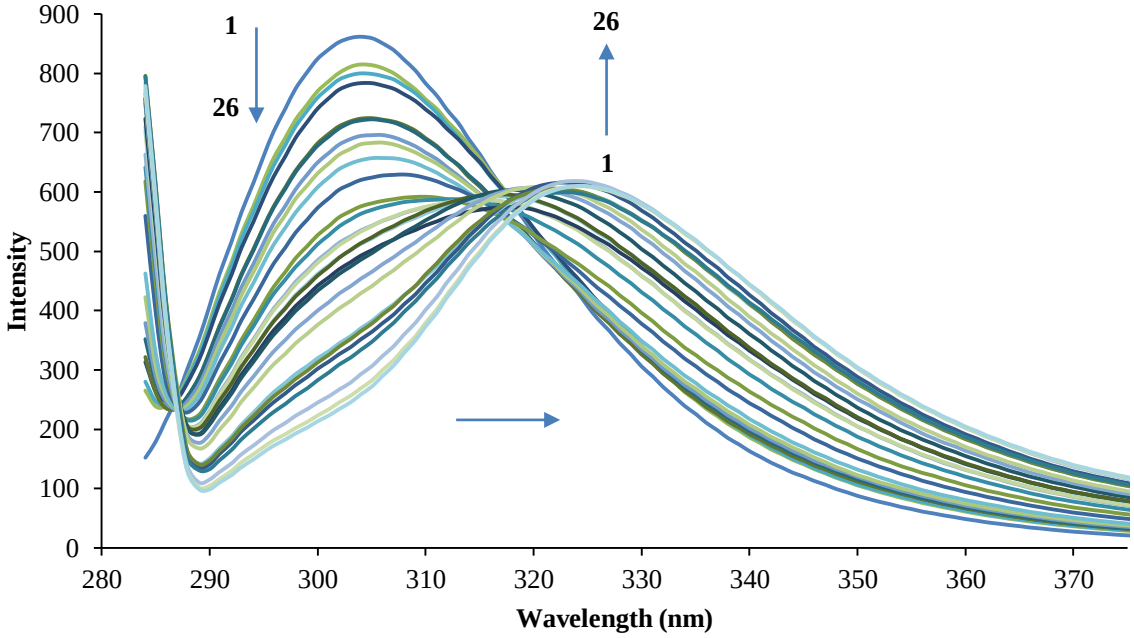

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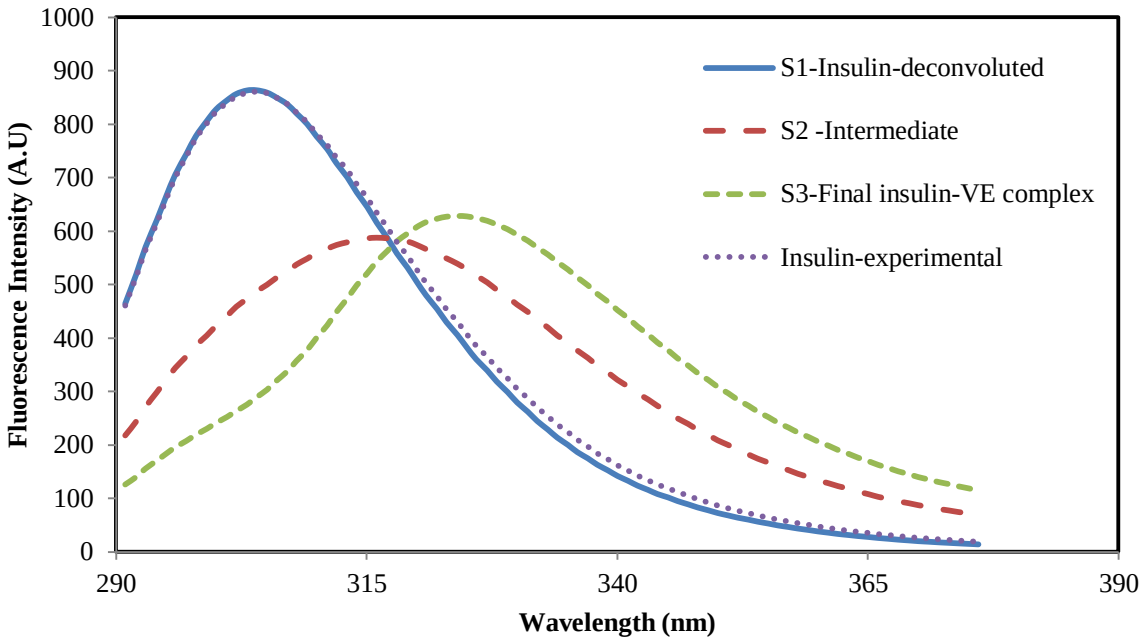

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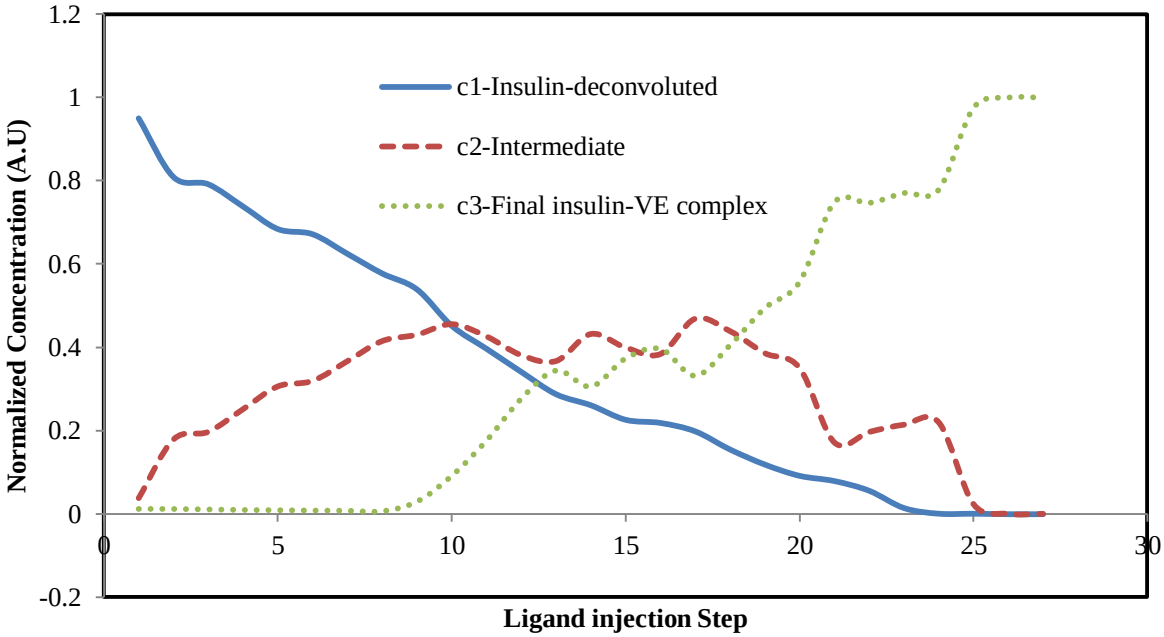

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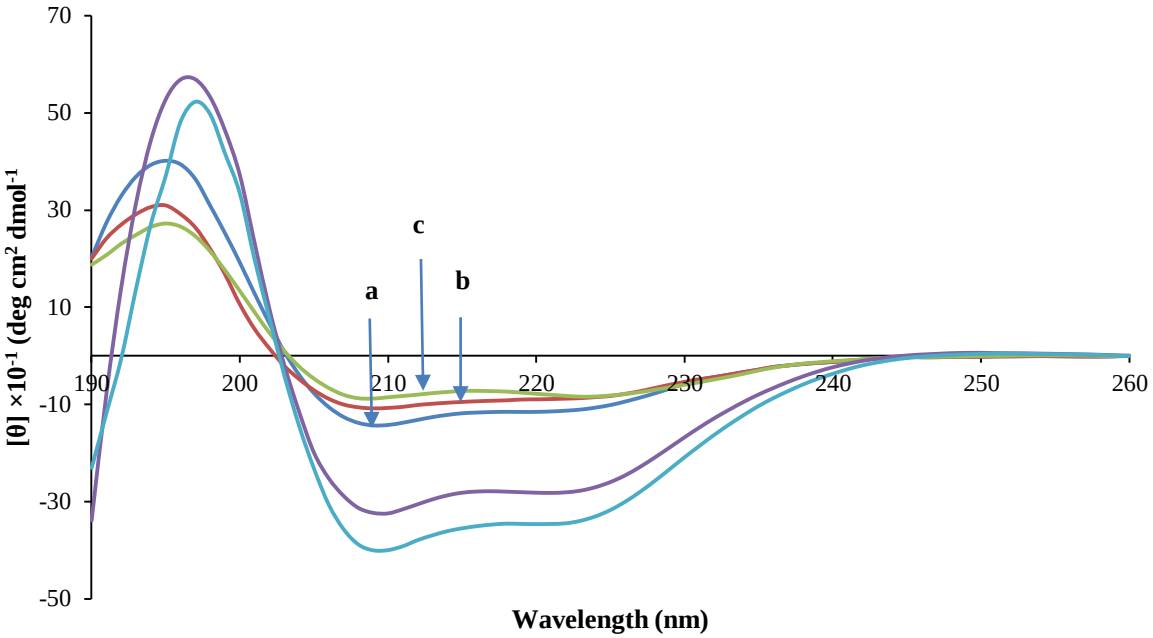

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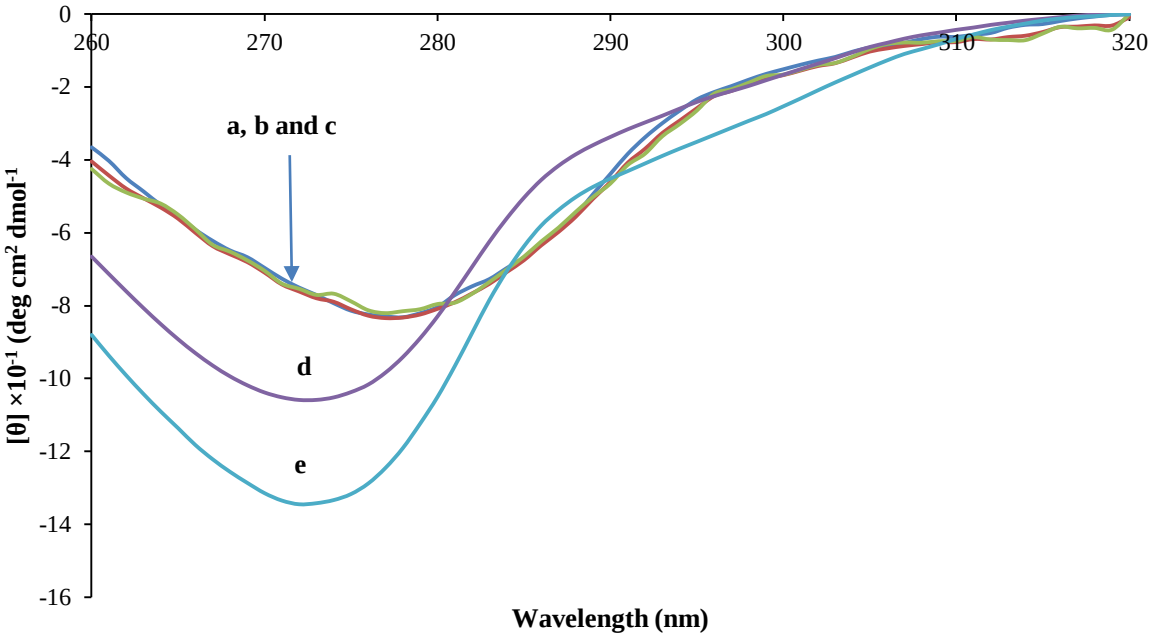

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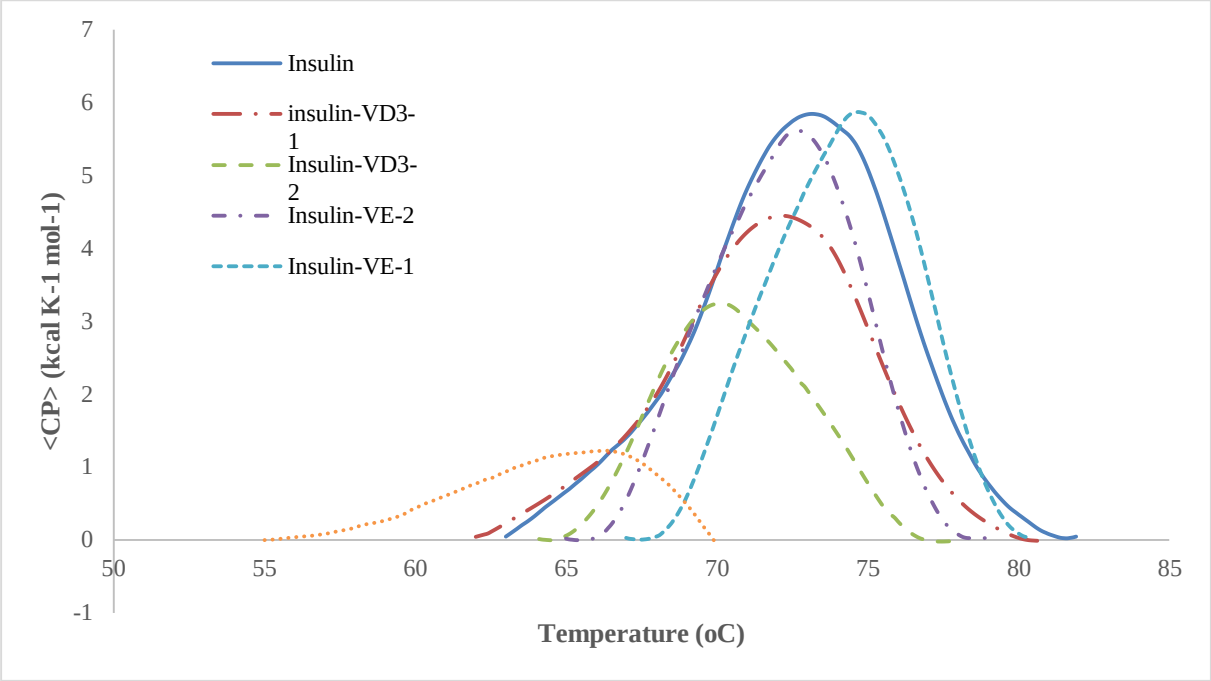
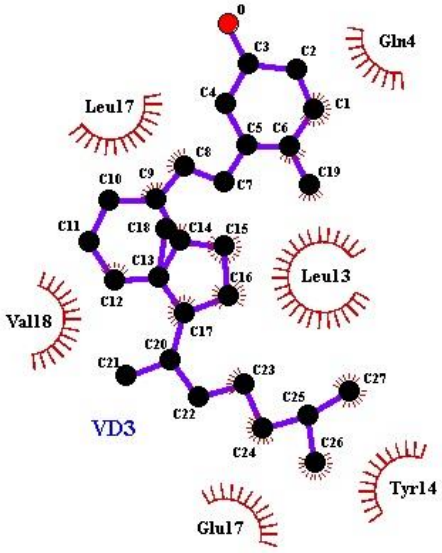
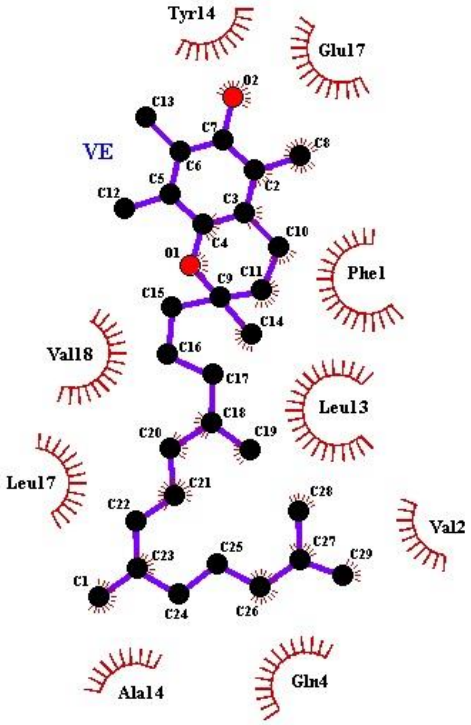


Fig. 4. (a)



Insulin-VD3 complex

Fig. 4. (b)



Insulin-VE complex

Fig. 5

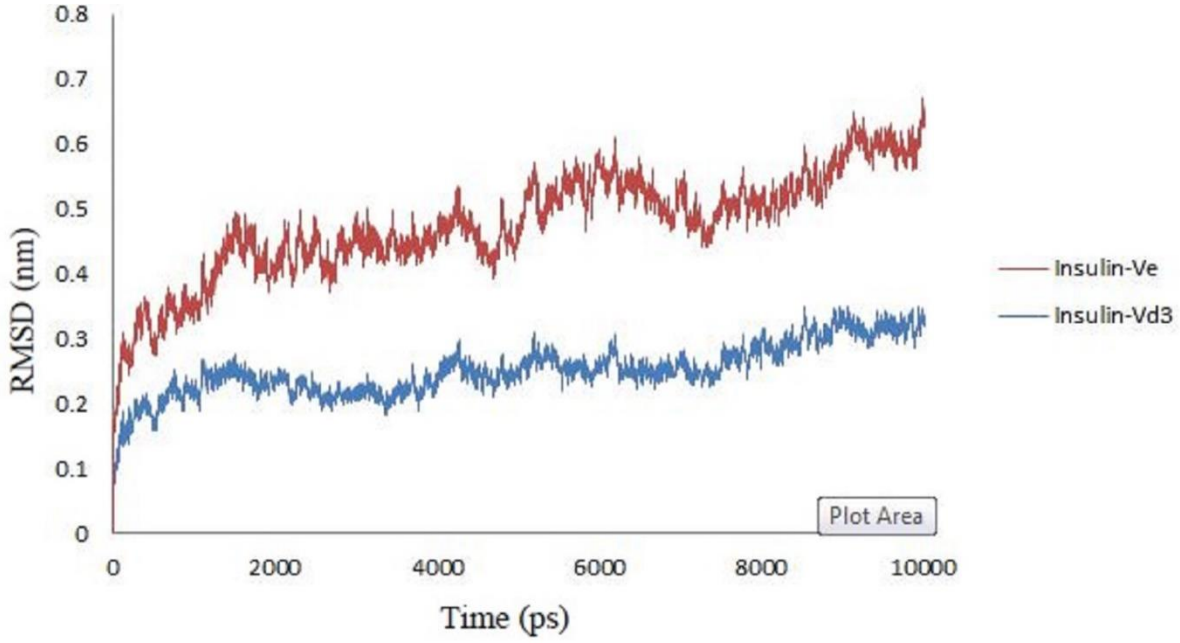


Fig. 6 (a)

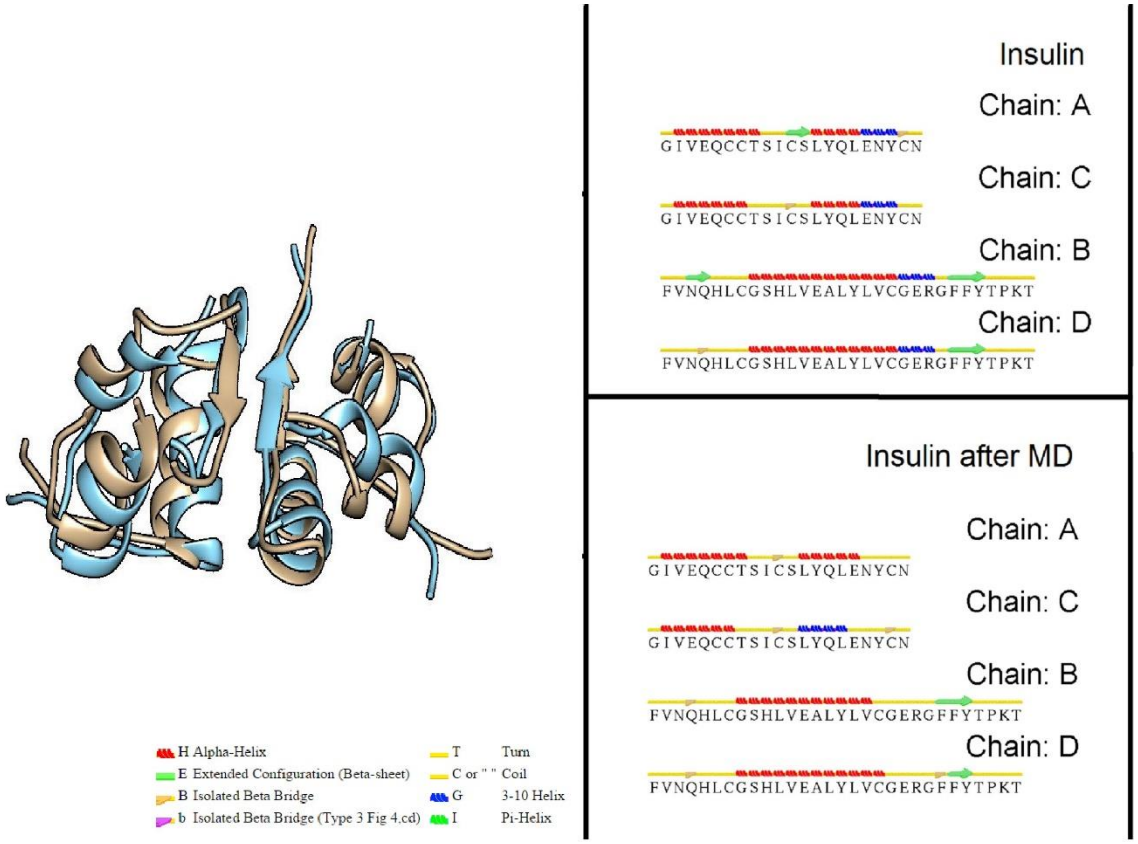


Fig. 9 (b)

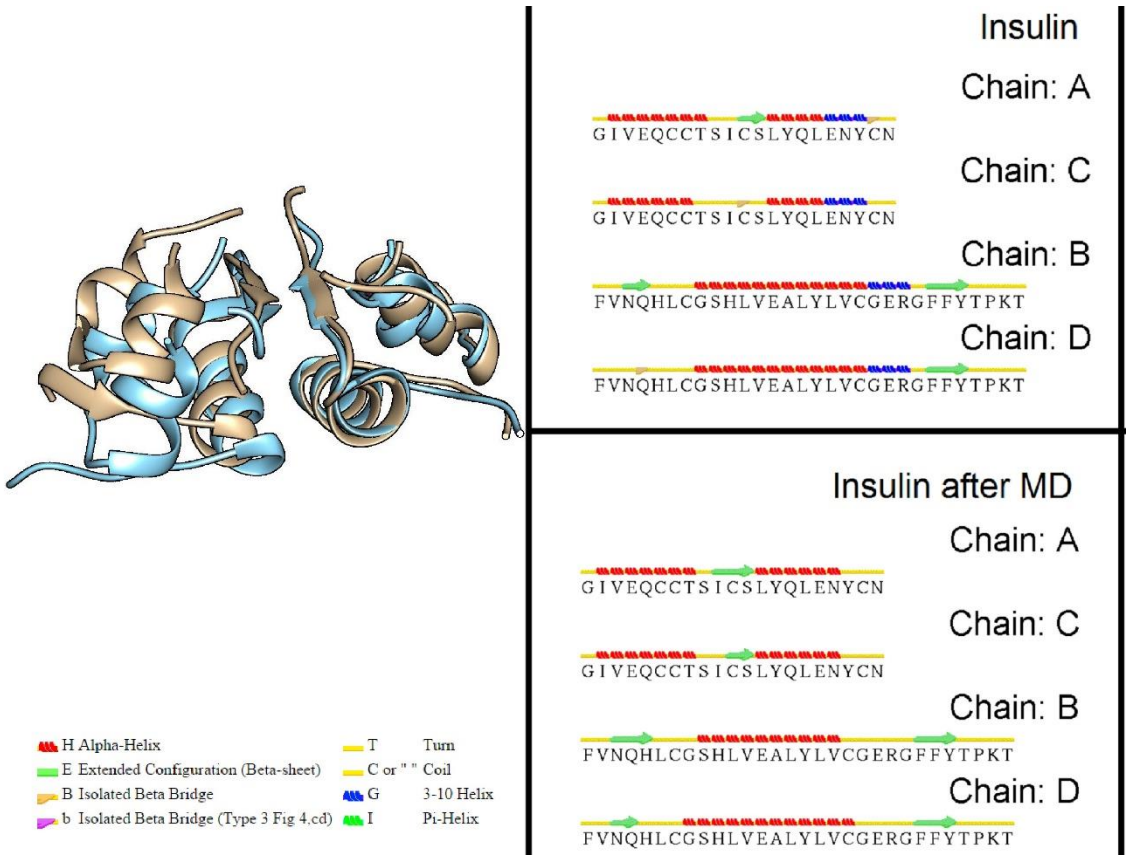


Fig. 7

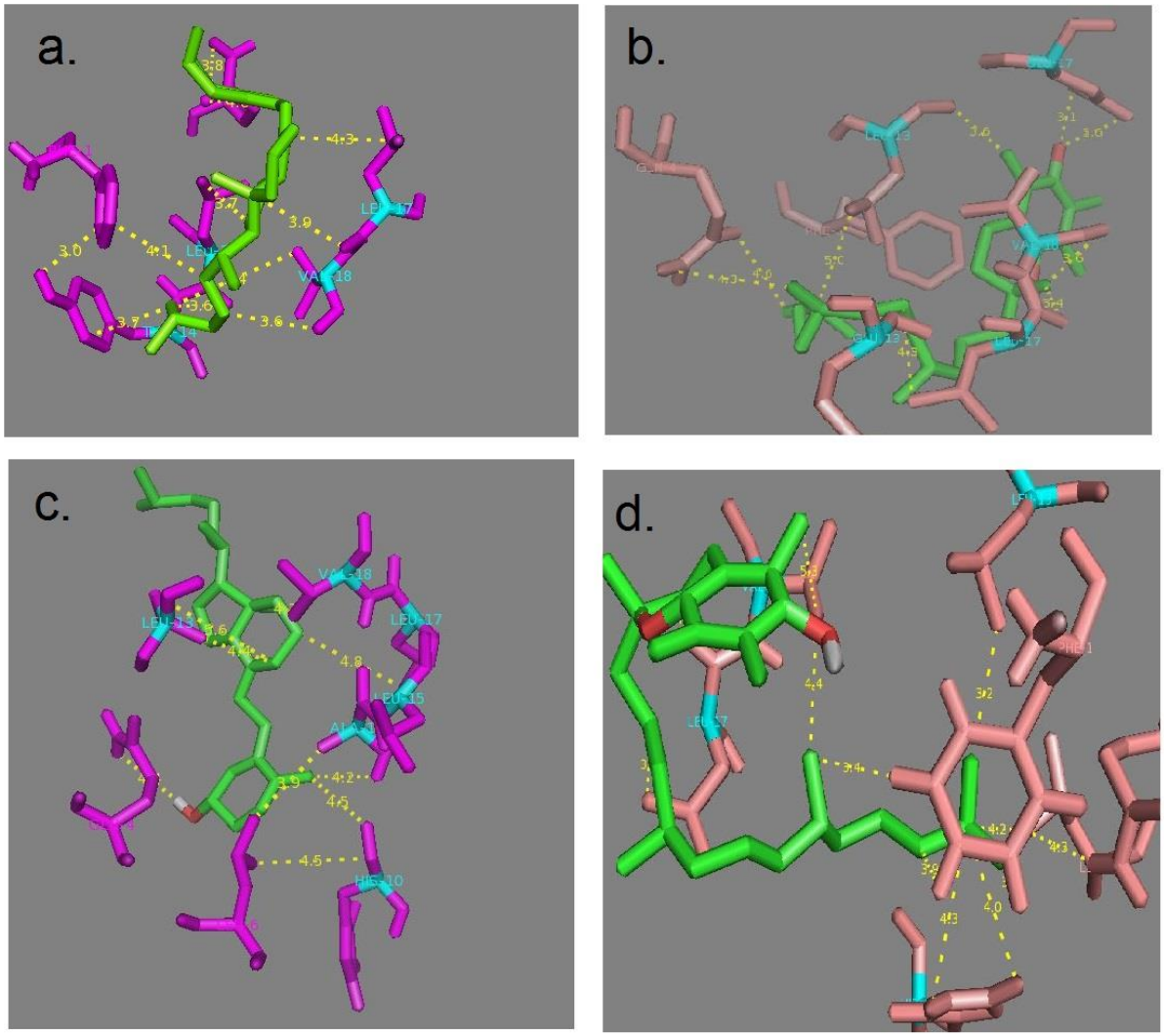


Table 1. Various parameters of human insulin upon interaction with vitamin D_3 and α-tocopherol.

Complex	K_{SV} ($\times 10^5$ M^{-1})	K_b ($\times 10^5$ M^{-1})	n	f_a	ΔG^o (kcal mol^{-1})
Insulin-VD_3	10182	2.7 ± 0.12	1.3 ± 0.034	1.35	-7.4
Insulin-VE	12096	1.5 ± 0.14	1.2 ± 0.027	1.52	-7.04

Table 2. Thermodynamic parameters obtained from the DSC method for human insulin in the absence and presence of vitamin D_3, α-tocopherol and ethanol.

Samples	ΔH^o (kcal mol^{-1})	ΔS^o (kcal K^{-1} mol^{-1})	T_m (^oC)	ΔC_p (kcal K^{-1} mol^{-1})	ΔG^o (kcal mol^{-1})	% Reversibility
Ins	47.8	0.138	73.1	0.64	8.89	9.8
[VD_3]/[ins]=4.35	36.0	0.104	72.2	0.51	6.64	8
[VD_3]/[ins]=8.70	20.2	0.103	70.1	0.39	3.85	7.4
[VE]/[ins]=1.35	36.7	0.105	74.7	0.6	7.49	9.9
[VE]/[ins]=2.70	35.2	0.102	72.7	0.57	6.83	9.6
Ethanol + Ins	9.4	0.028	66.3	0.32	1.98	5.1