Partial Purification of Dipeptidyl Peptidase-4 From Human Blood Serum and Study the Inhibitory Effect of Some Nitrile Derivatives on it

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Abstract

The research includes the partial purification of dipeptidyl peptidase-4 (Dpp-4) from a person’s natural serum. The enzyme was purified in three steps, precipitation by ammonium sulfate (50%), dialysis and gel filtration by Sephadex G-100. The results showed a significant increase of Dpp-4 activity and specific activity, and a decrease in total activity after purification. The descent of the purified enzyme with the highest activity from the separation column was identical to the descending of the protein with the highest activity. The inhibitory activity of nitrile derivatives that were used on the purified enzyme was studied. Nitrile derivatives were used to inhibit the activity of the Dpp-4, selected based on similarity in sites of effective groups between derivatives and anti-hyperglycemic drugs (Dpp-4 inhibitors type). The results show an inhibitory effect by the two nitrile derivatives with different concentrations. These inhibitors can work to reduce the level of blood sugar in people with diabetes. Moreover, Molecular docking was exerted to understand interaction mechanism of the compounds against Dpp-4 at atomic level based on experimental processes.

Keywords: Dipeptidyl peptidase-4, molecular docking, nitrile, gel filtration

Introduction

Diabetes is a clinical metabolic disease that causes an increase in the level of glucose in the blood (hyperglycemia) which occurs as a result of an imbalance in the cell’s reception of insulin or a lack of metabolism of insulin [1]. The use of treatment for diabetics from the first moment of its discovery is very necessary because it aims to control the development of the disease and therefore no complications or any side effects are resulting from these complications, whether diet, exercise, or the use of medicines[2]. Dipeptidyl peptidase-4 (Dpp-4) is a type of serine protease, which disrupts incretin hormones such as glucagon peptide-1 (GLP-1) and glucose-dependent insulin peptide (GIP), which were secreted from endocrine cells in response to the presence of food in the small intestine[3]. Dipeptidyl peptidase-4 also has nonglycemic effects by controlling the progression of inflammation, which may be mediated more through direct protein-protein interactions than catalytic activity in the context of nonalcoholic fatty liver disease, obesity and type 2 diabetes T2DM [4]. The Dpp-4 enzyme is present in many human tissues, including the pancreas, fat cells, liver, and as proteases present on the cell surface [5]. Some studies have reported associations of Dpp-4 with infection or attenuation of lung injury [6,7] Others have discovered numerous Dpp4 transcripts in the nasal epithelia of children and revealed the up-regulation of CD26 on human T lymphocytes upon a correlation with soluble Dpp4 activity [8]. One of the new types of drugs used to lower blood sugar of T2DM is dipeptidyl peptidase inhibitors (Dpp-4) by designing rational drugs, based on an understanding of the underlying mechanism of action and knowledge of the target structure of an

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They stimulate insulin secretion and inhibit glucagon secretion by elevating endogenous GLP-1 concentrations without an intrinsic hypoglycemia risk. Their activity potential to lower HbA1c is in the range between 0.5 and 1.0% and their safety profile is favorable. Dpp-4 inhibitors are body weight neutral and they have demonstrated cardiovascular safety. In people with diabetes, the Dpp-4 inhibitor can prevent DDP-4 to mitigate the degradation of GLP-1, prolong its effect and raise insulin activity for the purpose of lowering blood glucose. However, the negative effects of Dpp-4 inhibitors severely hamper their clinical applications, especially since there is a clinical demand for new Dpp-4 inhibitors from various sources including chemical composition, herbs and plants with fewer side effects. Through modern chemical technologies and some computer-based research methods such as virtual screening, molecular dynamics simulation, and molecular docking new strategies can be built to discover and design new inhibitors. In the meantime, molecular modeling is also directly related to measurable biological structures and quantities. For these reasons, molecular docking studies are performed to get insights at atomic level for treatment of the disorders as well as diseases.

**Materials and Methods**

Ammonium sulfate (AS) with a purity of ≥99%, KH₂PO₄ and K₂HPO₄ with 99% purities were purchased from Fluka, Sephadex G-100 was purchased from Sigma Aldrich and Dpp-4 assay kit was purchased from Sigma Aldrich. 1-(3-mercapto-2-methylpropanoyl) pyrrolidine-2-carbonitrile (inhibitor 1) and 5N-(acetonitrile)-1,1-dimethyl biguanide (inhibitor 2) were synthesized. Auto Dock 4.2 program was used for molecular docking.

**Preparation of potassium phosphate buffer (50mM, pH 7.0)**

This phosphate buffer was prepared by dissolving 0.87 g of K₂HPO₄ in 100ml distilled water and 0.68 g of KH₂PO₄ in 100 ml distilled water separately, then 61.5 ml of K₂HPO₄ was mixed with 38.5 ml of KH₂PO₄ solution, pH was adjusted to 7.0, and the volume was completed to 200 ml with distilled water.

**Sampling:** Five ml of venous blood was drawn from 15 healthy people, in plain tubes and left at room temperature to complete the clotting process, the serum was separated after centrifugation at 3000 rpm for 20 minutes, stored at 20 °C until the next step.

**Purification of Dpp-4**

Precipitation of enzyme by ammonium sulfate

Solid ammonium sulfate was gradually added to 40 ml of crude enzyme (serum) until saturation reach 50% at 4 °C. The component was mixed gently for 45 min, then it was centrifuged at 6000 rpm for 20 min., the supernatant was discarded and the precipitate was dissolved in a suitable volume of potassium phosphate buffer (50 mM, pH 7.0). The activity of the enzyme was measured using Dpp-4 kit (Sigma Aldrich Assay kit) and protein concentration was determined according to the method of Bradford.

**Dialysis**

The enzyme solution was dialyzed after precipitation with ammonium sulfate against distilled water for 24 h under cooling conditions (4 °C) with stirring and changing the D.W for four times, then the enzyme activity, protein concentration, specific activity was measured.

**Dpp4 purification by gel filtration chromatography**

Sephadex G-100 was prepared as recommended by Pharmacia Fine Chemicals Company. A quantity of Sephadex G-100 was suspended in 0.05 M phosphate buffer (pH 8), degassed, and packed in a glass column (1.5×30cm), then equilibrated with the same buffer. Concentrated of Dpp-4 obtained from ammonium sulfate step was applied onto the column. Elution was achieved at a flow rate of 30 ml/h (2 ml/fraction) and the same buffer was used for equilibration. The column flow velocity was very controlled to obtain more accurate product. The absorbance of each fraction was measured at 280 nm. Dpp-4 was also determined in each fraction and protein concentration was determined using Bradford method.

**Determination of various inhibitors effect on enzyme activity**
The enzyme was incubated with an equal volume of different inhibitors 1-(3-mercapto-2-methylpropanoyl) pyrrolidine-2-carbonitrile (inhibitor 1) and 5N-(acetonitrile)-1,1-dimethylbiguanide (inhibitor 2), using different concentrations 10^{-2} M, 10^{-3} M, 10^{-4} M and 10^{-5} M at 37 °C for 30 minutes. The enzyme activity was assayed for each treatment concentration. The control was the enzyme solution without any of these compounds. The remaining activity was assayed for each treatment.

**Molecular docking**

Besides experimental procedures, the interaction mechanisms of inhibitors 1 and 2 as ligands with Dpp-4 as targets were investigated using AutoDock 4.2\(^{[13]}\) at the atomic level based on molecular docking technique. Firstly, ligand(s) are prepared using Gaussian 09 (G09)\(^{[11]}\) as well as target enzyme is prepared in Discovery Studio (DS) 2020\(^{[8]}\) for molecular docking study. The protein crystal structure (PDB code: 4A5S) has been obtained from protein bank database. The binding site is defined from PDB sites records by using DS 2020 software. During the docking analysis, the ligands are flexible and Dpp-4 enzyme is held rigid. All the docking processes are also applied with the default settings.

**Results**

As mentioned in experimental part, many techniques were used to purify normal human serum Dpp-4. The purification of the profile of Dpp-4 is summarized in Table 1, the activity and specific activity of the crude enzyme were 25.8 U/ml and 25.2 U/mg respectively, in addition to that, the precipitation of crude enzyme by AS (50%) increased the activity and specific activity to 33.6 U/ml and 28 U/mg respectively, and decreased the total activity to 1008 U, at a recovery of 78%. Upon dialysis, the total activity of Dpp-4 decreased from 1008 U to 1002 U, while activity and specific activity increased to 40.1 and 57.2 U/mg respectively, and about 77.6% yield. Finally, after gel filtration, the activity and specific activity were increased to reach 45.3 U/ml and 151 U/mg respectively, and the total activity decreased to 906 U at a recovery of 70%. These results were in agreement with previous studies which showed a significant increase of Dpp-4 activity\(^{[9]}\). An attempt to improve the amount of AS% saturation was achieved by gradual experimentation (incremental increase) until the best saturation ratio was obtained. The specific activity is an indication of the degree of purification.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume* (ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activity (U/mg protein)</th>
<th>Total activity (U)</th>
<th>Purification (folds)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>50</td>
<td>25.8</td>
<td>1.02</td>
<td>25.2</td>
<td>1290</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>AS precipitation (50%)</td>
<td>30</td>
<td>33.6</td>
<td>1.2</td>
<td>28</td>
<td>1008</td>
<td>1.1</td>
<td>78</td>
</tr>
<tr>
<td>Dialysis</td>
<td>25</td>
<td>40.1</td>
<td>0.7</td>
<td>57.2</td>
<td>1002</td>
<td>2.2</td>
<td>77.6</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>20</td>
<td>45.3</td>
<td>0.3</td>
<td>151</td>
<td>906</td>
<td>5.9</td>
<td>70</td>
</tr>
</tbody>
</table>

*50 mL of human serum used

Figure 1 shows the elution profile of Dpp-4 which was obtained from gel filtration chromatography. The enzyme activity starts to increase gradually from fraction 10 to reach to the top in fraction 14 and returned to decrease gradually until the activity was lost in fractions 21-23. The sharp increase in enzyme activity was observed in fractions 13-18. Fractions 11-13 had the least protein content, while fractions 14-19 had the highest protein content, as shown in Figure 1.
Table 2 shows the effect of different concentration of the two inhibitors 1-(3-mercapto-2-methylpropanoyl) pyrrolidine-2-carbonitrile(inhibitor 1) and 5N-(acetonitrile)-1,1-dimethylbiguanide (inhibitor 2) on Dpp-4 activity. According to the results of the Table 2, the activity of the enzyme decreases with an increase in the concentration of inhibitors, that is, the activity is inversely proportional to the increase in the concentrations of inhibitors.

Table 2. The remaining activity of Dpp-4 after using inhibitors

<table>
<thead>
<tr>
<th>Concentrations of inhibitors</th>
<th>(Remaining activity) %</th>
<th>Inhibitor 1</th>
<th>Inhibitor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-2} M</td>
<td></td>
<td>45.8</td>
<td>37.2</td>
</tr>
<tr>
<td>10^{-3} M</td>
<td></td>
<td>64.5</td>
<td>51.1</td>
</tr>
<tr>
<td>10^{-4} M</td>
<td></td>
<td>80.0</td>
<td>71.4</td>
</tr>
<tr>
<td>10^{-5} M</td>
<td></td>
<td>88.2</td>
<td>82.4</td>
</tr>
<tr>
<td>Control without inhibitors</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The percentage inhibitions for the two inhibitors were calculated according to the following equation:

\[
\text{Inhibition percentage (\%) } = 100 - \left[ \frac{\text{Activity with inhibitor}}{\text{Activity without inhibitor}} \right] \times 100
\]
In addition to purification process, molecular docking was applied to estimate how the mentioned ligands may bind in the active site of the target and how also tight the binding interaction would be. In the other words, this section allowed us to characterize the behavior of the ligands in binding site of target, as well as to explain undefined parts and the basic biochemical processes of the purification stage.

Therefore, all inhibitors (1-2) are docked at the binding sites of Dpp-4. AutoDock 4.2 is used for all docking simulations. From the docking analysis, insights into the interactions between the compounds and the enzyme were gained. According to docking analysis, inhibitor 1,2 have the mentioned important interactions in active site of Dpp-4. The Hydrogen bonds, electrostatic, hydrophobic and other non-bond interactions present in Dpp-4 inhibitor (1,2) complexes are displayed in Figure 2. Especially, inhibitor 2 forms nine hydrogen bonds with Asp192, Asn170, Glu191 and Arg253 and also two electrostatic interactions with Glu191 at active site of the enzyme molecule, Table 3. In the meantime, inhibitor 1 forms five hydrogen bonds with Arg356, Phe357, Ile374, Gly355 and Arg358 residues and one hydrophobic bond with Arg358 in active site of Dpp-4, Table 3.

![Figure 2. Docking poses of inhibitor 1 (left side), and 2 (right side) in Dpp-4.](image_url)

**Table 3. Interactions types and distances of compounds (1 and 2) – Dpp-4 complexes.**

<table>
<thead>
<tr>
<th>Interactions</th>
<th>Distance Å</th>
<th>Bonding</th>
<th>Bonding Types</th>
<th>Binding site of target</th>
<th>Binding site of ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:ARG358 - :1</td>
<td>5.101</td>
<td>Hydrophobic</td>
<td>Alkyl</td>
<td>A:ARG358</td>
<td>:1</td>
</tr>
</tbody>
</table>
Table 4. Binding energy, root mean square deviation (RMSD) values of inhibitor 1–ACE and inhibitor 2–ACE complex

<table>
<thead>
<tr>
<th>Complex</th>
<th>Binding Energy, kcal/mol</th>
<th>RMSD, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor 1-Dpp-4</td>
<td>-6.09</td>
<td>0.0697</td>
</tr>
<tr>
<td>Inhibitor 2-Dpp-4</td>
<td>-8.67</td>
<td>0.00951</td>
</tr>
</tbody>
</table>

**Discussion**

Dpp-4 has been purified and from different sources, and by different methods [2]. Among the results obtained in Table 1, the increase in the activity of the enzyme and the specific activity of the enzyme indicates an increase in the activity of isolating the enzyme and purifying it from blood serum, where the more effective the enzyme with the progress of work the more pure we get an enzyme. The use of Sephadex G-100 in the process of purification of the enzyme with a chromatography column and using the gel filtration method that depends
on the descent of materials according to their molecular weights was very successful, as specific activity of the enzyme of 151 after it was 25.2 before using the column was obtained, as well as a decrease in the value of Total activity from 1290 to 906, which indicates the activity of the purification and isolation process, this results accepted with [4]. Dpp-4 inhibition plays an important role in the management of type 2 diabetes and as we mentioned earlier as Dpp-4 also performs many other processes and functions including apoptosis, cell proliferation, protein interactions, cell adhesion etc [8]. In this study, we selected newly manufactured nitrile derivatives as a Dpp-4 inhibitor [13,14], therefore, the main reason for choosing nitrile derivatives because the nitrile group is an effective group used in most gliptins treatment that is used recently as a preservative for blood sugar (antihyperglycemia) [14]. Depending on the results, the highest percentage of inhibition (54.22%) of the Dpp-4 was obtained at a concentration of 10^-2 molar for inhibitor 1, while the lowest inhibition percentage (11.77%) at concentration 10^-5 molar, in other words, that the percentage of inhibition was directly proportion to the concentration of the inhibitor 1 (Table 2). This was what happened with the inhibitor 2 also where it was a higher percentage of inhibition (62.76) at concentration 10^-2 molar and the lowest percentage inhibition (17.61) at concentration 10^-5 M. Finally, the molecular docking of the synthesized compounds was realized, and the results of such studies were summarized. In molecular docking studies revealed that inhibitor 2 for Dpp-4 have relatively optimal Binding Energy (-8.67 kcal/mol), and RMSD (0.00951) values as compared to other one. The obtained result from the docking process has supported the in vitro activity values.

**Conclusion**

This study shows us not only hydrogen bonds, but also electrostatic interactions are very significant effect to display inhibitor activity of any molecule. It also expresses the nitrile derivatives, inhibitor 1 and 2 are more effective based on the in vitro and modeling analysis. Hence, this information provides a useful insight for the development of novel Dpp-4 inhibitor compound(s) with specific selectivity.

**Conflict of Interest:** None

**Funding:** Self

**Ethical Clearance:** Not required

**References**


